

## p53 REVIEW ARTICLE

## TP53 and Liver Carcinogenesis

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Primary hepatocellular carcinoma (HCC) is one of the most common malignancies and has the fourth highest mortality rate worldwide. The major risk factors, including chronic infections with the hepatitis B or C virus, are exposure to dietary aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), vinyl chloride, or alcohol consumption. Southern China and sub-Saharan Africa have the highest dietary AFB<sub>1</sub> exposure, making it and hepatitis B virus (HBV) the major causes of cancer mortality in these geographic areas. Recent studies have discovered genetic and epigenetic changes involved in the molecular pathogenesis of HCC, including somatic mutations in the p53 tumor suppressor gene (TP53). AFB<sub>1</sub> induces typical G:C to T:A transversions at the third base in codon 249 of p53. Chronic active hepatitis B and C (HCV) infection, and further inflammatory and oxyradical disorders including Wilson disease (WD) or hemochromatosis, generate reactive oxygen/nitrogen species that can damage DNA and mutate the p53 gene. The X gene of HBV (HBx) is the most common open reading frame integrated into the host genome in HCC. The integrated HBx is frequently mutated and has a diminished ability to function as a transcriptional cotransactivator and to activate the NF-kappa B pathway. However, the mutant HBx proteins still retain their ability to bind to and abrogate p53-mediated apoptosis. In summary, both viruses and chemicals are implicated in the etiology and molecular pathogenesis of HCC. The resultant molecular changes in the *ras* and *Wnt* signal-transduction pathways, and the p53 and *Rb* tumor suppressor pathways significantly contribute to liver carcinogenesis. *Hum Mutat* 21:201–216, 2003. Published 2003 Wiley-Liss, Inc.†

KEY WORDS: cancer; carcinogenesis; hepatocellular carcinoma; p53; TP53; aflatoxin B<sub>1</sub>; HBV; HCV; vinyl chloride; oxidative stress; nitrosative stress; tumor exposure; mutagen; risk factor

## DATABASES:

TP53 – OMIM: 191170; GenBank: NM\_000546 (mRNA)

<http://p53.curie.fr/> (p53 Web Site at Institut Curie)

[www.iarc.fr/p53](http://www.iarc.fr/p53) (IARC p53 Mutation Database)

### INCIDENCE AND ETIOLOGY OF HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC), the major manifestation of primary liver cancer, is one of the most frequent and malignant diseases worldwide. With about 437,000 estimated new diagnosed cases per year, it is the fifth most common cancer (5.4% of all new cancer cases) [Parkin et al., 1999; Pisani et al., 1999]. While this malignancy is less common in Western developed countries such as the United States, with an incidence of 2.8 to 6.1 (Caucasian vs. African-American) per 100,000 [El-Serag and Mason, 1999], it is endemic in China, Taiwan, Korea, and sub-Saharan Africa, where the incidence is between 20 to 100 cases per 100,000 inhabitants (Figs. 1A, B) [Dominguez-Malagon and Gaytan-Graham, 2001; Sherman, 1995]. Furthermore, HCC is a leading cause of cancer-related death in these countries, because less than 3% of these patients survive more than 5 years [Di Bisceglie et al., 1988].

The major HCC risk factors include various chemicals and viruses, and are summarized in Figure 2. Among those, chronic HBV and HCV infections attribute to the HCC development in more than 80% of the HCC cases worldwide [reviewed in Chen et al., 1997]. Other known risk factors, including AFB<sub>1</sub> uptake, cigarette smoking, or heavy alcohol consumption are capable of inducing HCC alone, but they also have synergetic effects [reviewed in Chen et al., 1997]. Besides these risk factors there are several genetic disorders (Fig. 2), such as hemochromatosis (an iron overload disease) and Wilson disease (a copper overload disease) (WD), that are associated

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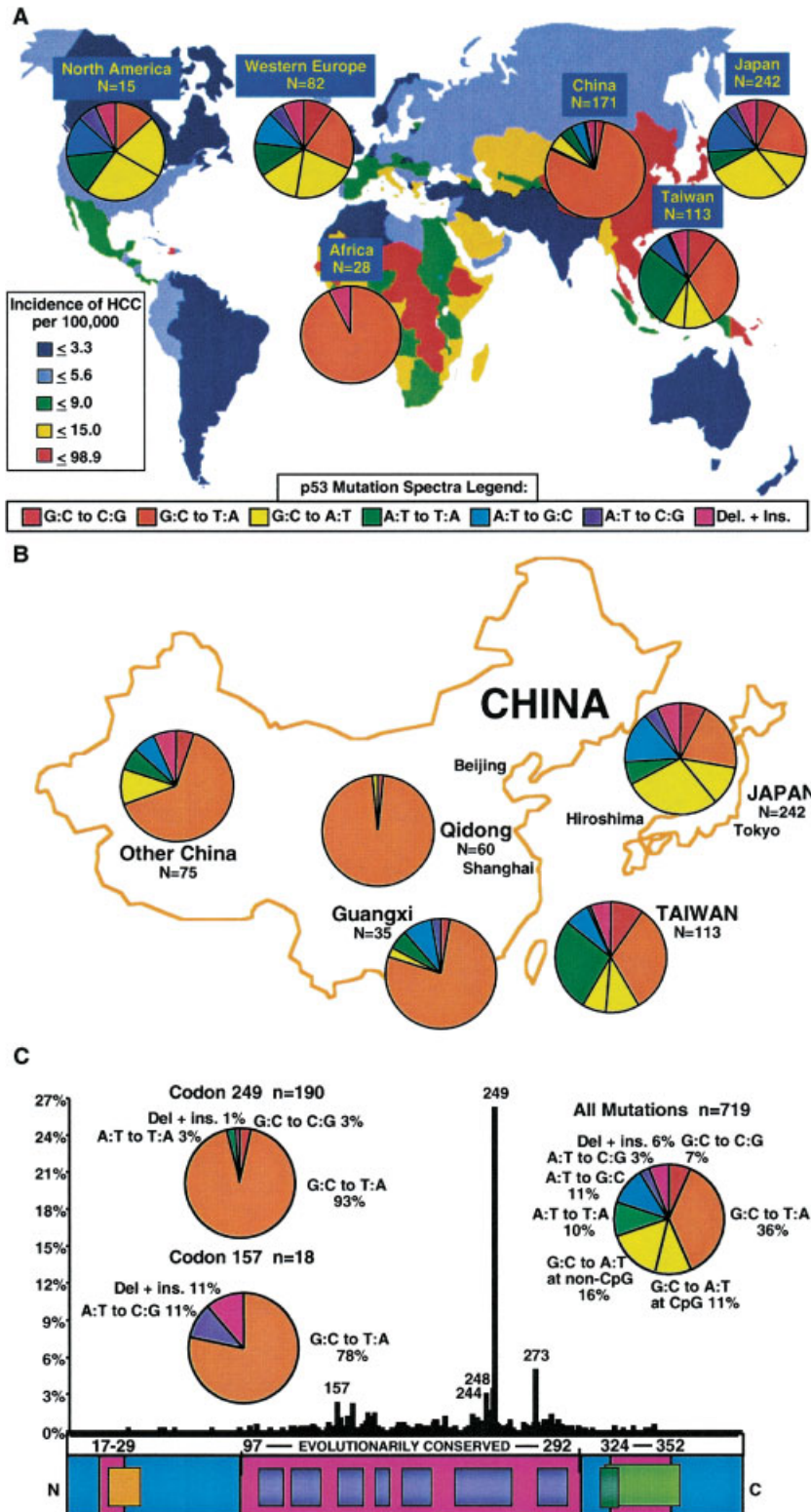
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with an increased risk of HCC [Hofseth et al., 2002]. Regardless of its etiology, cirrhosis alone is an independent risk factor for HCC.

In recent years, we have gained a better understanding of the genetic and environmental interac-

tions and mechanisms associated with the development of primary liver cancer. Recent studies have provided evidence that the *p53* tumor suppressor gene (TP53; MIM# 191170) plays a major role in hepatocarcinogenesis.



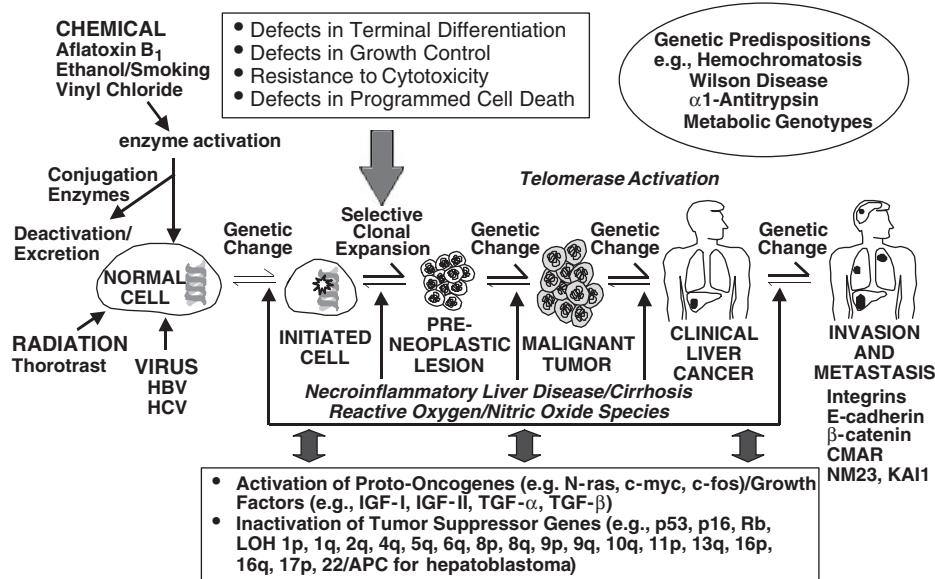


FIGURE 2. Multistage hepatocarcinogenesis. (CMAR, cellular adhesion regulatory molecule; HBV, hepatitis B virus; HCV, hepatitis C virus; IGF-I, insulin-like growth factor-I; IGF-II, insulin-like growth factor-II; LOH, loss of heterozygosity; TGF- $\alpha$ , transforming growth factor  $\alpha$ ; TGF- $\beta$ , transforming growth factor  $\beta$ ).

Besides *p53*, several oncogenes and tumor suppressor genes are discussed as showing involvement in HCC. Oncogenes like *N-ras*, *c-myc*, or *c-fos* have been

found to be overexpressed, but their mutations are rare and, so far, there is only little evidence for a direct implication in hepatocellular carcinogenesis [Hofseth et al., 2002; Tabor, 1994]. Recently, an inappropriate reactivation of the *Wnt*-pathway, resulting from  $\beta$ -*catenin* gene alterations, has been implicated in liver carcinogenesis [Wong et al., 2001, reviewed in Buendia, 2000].  $\beta$ -*catenin* mutations are found in 18–41% and the nuclear overexpression occurs in 10–90% in HCC. This deregulation of cellular  $\beta$ -*catenin* may transcriptionally activate the target genes of the *Wnt*-pathway, e.g., *c-myc*, *cyclinD1*, and *PPAR $\delta$* , and thus, may promote tumor progression by stimulation of cell proliferation [Buendia, 2000; Nhieu et al., 1999; Wong et al., 2001]. In addition to  $\beta$ -*catenin*, abnormalities of the retinoblastoma tumor suppressor gene (*Rb*) have been reported in advanced HCC [reviewed in Buendia, 2000; Hofseth et al., 2002]. *Rb* gene mutations are found in 20 to 25% as a single gene mutation, but together with *p53* mutations they have been found in 80 to 86% of HCC [Tabor, 1994]. Both tumor suppressor genes might additionally contribute to hepatocarcinogenesis, because *p53* malfunctions would most likely result in *Rb* gene activation in order to suppress cell growth and tumor formation [Dominguez-Malagon and Gaytan-Graham, 2001].

This review will provide some insight into the various functions and involvement of the *p53* tumor suppressor gene during primary liver cancer development.

### TP53 MUTATION SPECTRUM IN HCC

Liver carcinogenesis is a multistep and multifactorial process driven by genetic or epigenetic

FIGURE 1. **A:** Worldwide incidences of HCC with pie diagrams of *p53* mutation patterns for various countries and continents according to the IARC *p53* mutation database. ([www.iarc.fr/p53/index.html](http://www.iarc.fr/p53/index.html)). A high frequency of G:C to T:A mutations is found in high incidence areas of both endemic HBV infection and dietary AFB<sub>1</sub> exposure. **B:** *p53* mutational spectra in HCC from different geographic areas of Asia. *p53* 249<sup>ser</sup> mutations are found only in high incidence geographic areas with endemic HBV infections and dietary AFB<sub>1</sub> exposure. **C:** Schematic representation of the *p53* molecule. The human *p53* protein consists of 393 amino acids with functional domains, evolutionarily conserved domains, and regions designated as mutational hotspots. Functional domains include the transactivation region (gold block), sequence-specific DNA-binding region (amino acids 100–293), nuclear localization sequence (amino acids 316–325, dark green block), and oligomerization region (amino acids 319–360, light green block). Evolutionarily conserved domains (amino acids 17–29, 97–292, and 324–352; rose-colored areas) were determined using the Multiple Alignment Construction and Analysis Workbench (MACAW) program. Seven mutational hotspots and evolutionarily conserved regions within the large conserved domain are also identified (amino acids 130–142, 151–164, 171–181, 193–200, 213–233, 234–258, and 270–286, dark blue blocks). Vertical lines above the schematic represent missense mutations. The majority of these mutations are in the conserved hydrophobic midregion of the protein, which is required for the sequence-specific binding to DNA. The nonmissense mutations (nonsense, frameshift, splicing, and silent) are distributed throughout the protein and determined primarily by sequence context, while deletions and insertions are more common in the N- and C-terminus of the *p53* gene. The pie charts on the left show the mutational spectra of codons 157 and 249, and the frequency evoked by AFB<sub>1</sub> intake. In contrast, the pie chart on the right includes all *p53* mutations in HCC.

TABLE 1. Assessment of Causation by the Bradford-Hill criteria

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Hypothesis: Dietary AFB<sub>1</sub> exposure can cause 249<sup>ser</sup> (AGG → AGT) p53 mutations during human liver carcinogenesis

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**Strength of association**

- Consistency
  - Positive dose-response correlation between estimated dietary AFB<sub>1</sub> exposure and frequency of 249<sup>ser</sup> p53 mutations in three different ethnic populations on three continents
  - 249<sup>ser</sup> p53 mutant DNA is detected in sera from individuals exposed to AFB<sub>1</sub> and infected with HBV
  - 249<sup>ser</sup> p53 mutations are found in HCC from individuals both exposed to dietary AFB<sub>1</sub> and infection with HBV but not with HBV alone
- Specificity
  - 249<sup>ser</sup> p53 mutations are uncommon in other cancer types
- Temporality
  - 249<sup>ser</sup> p53 mutant cells are observed in nontumorous liver in high HCC incidence geographic areas

**Biological plausibility**

- AFB<sub>1</sub> is a potent mutagen and carcinogen in laboratory studies
  - AFB<sub>1</sub> is enzymatically activated by human hepatocytes and the 8,9-AFB<sub>1</sub> oxide binds to the 3<sup>rd</sup> base (G) in codon 249
  - AFB<sub>1</sub> exposure to human liver cells in vitro produces codon 249<sup>ser</sup> p53 mutations
  - HBx gene expression increases the frequency of 249<sup>ser</sup> p53 mutations in cells exposed to AFB<sub>1</sub> in vitro
  - 249<sup>ser</sup> p53 expression inhibits apoptosis and p53 mediated transcription, and enhances liver cell growth in vitro
- 

damage in susceptible cells. During tumor initiation, the cells show various DNA mutations, gain an altered responsiveness to their microenvironment, and gain a selective growth advantage (Fig. 2) [reviewed in Elmore and Harris, 1998]. The p53 response pathway is frequently defective in human cancers including HCC. The frequency of p53 mutations and the mutation spectra vary in different geographic areas (Figs. 1A-C), and presumably reflect differences in both etiological agents and host susceptibility factors.

#### CAUSATION EVALUATED BY THE BRADFORD-HILL CRITERIA

The presence of specific p53 mutational hotspots in different types of human cancer implicates environmental carcinogens and endogenous processes in the etiology of human cancer. The presence of a characteristic p53 mutation can also manifest a molecular link between the exposure to a particular carcinogen and a specific type of cancer, e.g., AFB<sub>1</sub> exposure and codon 249<sup>ser</sup> mutations in HCC (Figs. 1B, C). In addition, the detection of such a particular mutation in normal-appearing tissue provides further support for the involvement of a specific carcinogen in a specific human cancer. Once a careful assessment of causation has been done for a presumed carcinogen association, its associated molecular damage, and the final disease, individuals with an increased cancer risk may be identified. For this approach, appropriate criteria as proposed by Bradford-Hill, might be used for the assessment [Hill, 1965]. Using the “weight of the evidence” principle, these criteria include strength of association (consistency, specificity, and temporality) and biological plausibility. One prime example for the use of the Bradford-Hill criteria is the association of AFB<sub>1</sub> dietary intake followed by p53 249<sup>ser</sup>

mutations and liver carcinogenesis (Table 1) [Hussain and Harris, 1998]. However, geographic location, race, genetic polymorphisms, and gender may also influence an individual’s susceptibility for cancer risk.

#### FACTORS CONTRIBUTING TO HCC Aflatoxin B<sub>1</sub>

p53 mutations indicate that the sites and features of DNA base changes differ among the various human tumor types. In human HCC, a unique mutational spectrum has provided a strong molecular link between carcinogen exposure and cancer development. In geographical areas, like China and Africa, where AFB<sub>1</sub> dietary exposure (by consuming mycotoxin-contaminated foods like corn, rice, and peanuts) and chronic viral hepatitis were found, a point mutation at the third position of codon 249 resulting in a G:C to T:A transversion was common in HCC (Figs. 1A-C) [Bressac et al., 1991; Hsu et al., 1991]. These results have been confirmed and extended by others [Li et al., 1993; Scorsone et al., 1992]. This codon 249 mutation can also be detected in serum DNA from patients from the Gambia with HCC, so that p53 mutant DNA may be a biomarker of exposure to AFB<sub>1</sub> and possibly early HCC [Kirk et al., 2000]. Furthermore, the p53 mutation load of 249<sup>ser</sup> mutant cells in nontumorous liver has a dose-dependent relationship with the intake of AFB<sub>1</sub> [Aguilar et al., 1994].

Several studies clearly support the findings of a positive correlation between the 249<sup>ser</sup> mutation of the p53 tumor suppressor gene and AFB<sub>1</sub> exposure, while the analysis of HCCs in areas of low AFB<sub>1</sub> intake always reported a different mutational spectrum [Kress et al., 1992; Oda et al., 1992; Ozturk, 1991]. In-vitro studies exposing human liver cell lines to AFB<sub>1</sub> presented the same 249<sup>ser</sup> mutational pattern

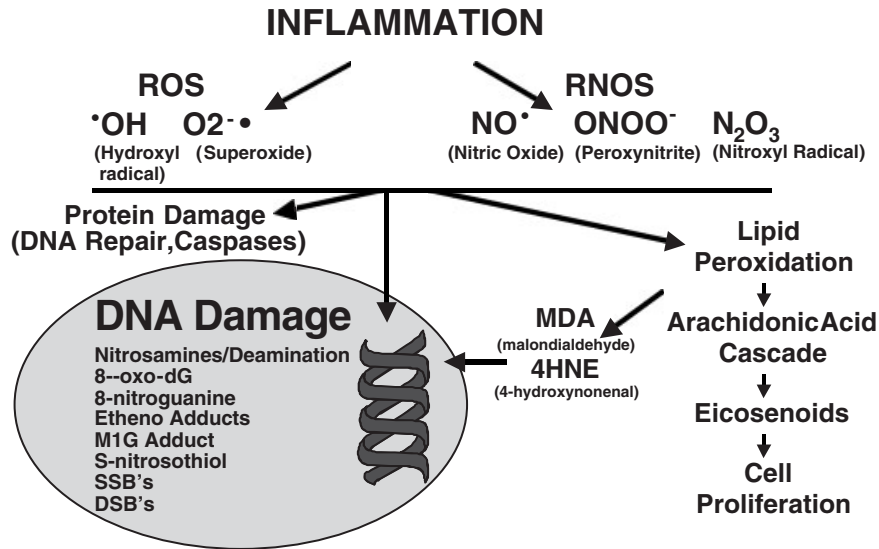


FIGURE 3. Overview of different reactive oxygen and nitrogen oxide species (ROS/RNOS) and their reaction products leading to DNA damage during inflammation. 8-oxo-dG, 8-hydroxy-deoxy-guanosine; SSB's, Single strand breaks; DSB's double strand breaks.

of the *p53* gene [Aguilar et al., 1993; Mace et al., 1997] as has been reported in the epidemiological studies (Fig. 1C).

Taken together, these results indicate that these mutational hotspots are specific for hepatocellular liver tumors and that genomic HBV integration is not necessarily required, which will be discussed later in this review [Hsu et al., 1993]. There are at least two possible explanations for these findings. Aguilar et al. [1994] have shown a higher relative abundance of the 249<sup>ser</sup> mutant liver cells in nontumorous liver by using a highly sensitive genotypic mutation assay. They suggested that this early mutational event may be due to the high mutability of the third base at the codon 249 to AFB<sub>1</sub>, as suggested by previous in-vitro studies in human liver cells [Aguilar et al., 1993; Cerutti et al., 1994]. Another possible explanation might be that the 249<sup>ser</sup> mutant *p53* protein may provide a special growth and/or survival advantage to these liver cells [Puisieux et al., 1995].

The latter hypothesis is supported by the following findings: 1) transfection of a *p53* 249<sup>ser</sup> mutant into *p53* negative human liver cancer cells resulted in enhanced cell growth [Ponchel et al., 1994]; 2) introduction of a murine *p53* mutation, corresponding to human codon 249 into a murine hepatocyte cell line, resulted in a selective growth advantage [Dumenco et al., 1995]; 3) the 249<sup>ser</sup> mutant inhibits wild-type *p53*-mediated apoptosis, resulting in increased cell survival [Wang et al., 1995]; and 4) the 249<sup>ser</sup> mutant is more effective than other mutants in inhibiting wild-type *p53* transactivation activity in human liver cells [Forrester et al., 1995].

One model for the liver carcinogenesis with a *p53*<sup>ser</sup> mutation is the metabolic activation of the AFB<sub>1</sub> to

8,9-epoxide that binds to DNA and results in pro-mutagenic N7dG adduct formation [Buss et al., 1990; Guengerich et al., 1996]. The underlying mechanisms are reviewed in more detail by Elmore et al. [Elmore and Harris, 2002] and Dominguez-Malagon et al. [Dominguez-Malagon and Gaytan-Graham, 2001a]. Together with an enhanced cell proliferation, e.g., due to chronic active hepatitis, these mutagenic N7dG adducts might then allow the fixation of the G:C to T:A transversion at codon 249 of the *p53* gene and a selective clonal expansion of the involved cells (Fig. 3).

All these observations suggest that the *p53* tumor suppressor gene plays a key role during early events of hepatocarcinogenesis in these geographic areas of high HCC incidence. However, in areas with minimal AFB<sub>1</sub> intake and low HCC incidence, *p53* mutations may occur as a later event in tumor progression [Hosono et al., 1993; Jaskiewicz et al., 1995; Teramoto et al., 1994]. For example, Tanaka et al. [1993] have reported that in some cases, malignant cells within HCC acquire *p53* mutations and that these cells then develop into dedifferentiated subpopulations within that HCC. This is in accordance with the study from Oda et al. [1994] showing different *p53* mutations in nodule-in-nodule HCCs as evidence for their association with HCC progression from an early to an advanced stage. Another finding linking mutant *p53* to the progression of liver cancer is that HCC containing areas with loss of heterozygosity (LOH) of the *p53* tumor suppressor gene were associated with more severe cellular atypia compared with those areas without *p53* LOH [Teramoto et al., 1994]. Finally, *p53* mutations preferentially occur in moderately and poorly differentiated HCC in association with or after

*p53* LOH [Oda et al., 1992] or in smaller HCC (*p53* mutations (positively correlated with Edmonson's grading) and lead to dedifferentiated subpopulations within this individual HCC [Tanaka et al., 1993].

### Vinyl Chloride

Another carcinogen leading to typical *p53* mutations was published for the first time in 1974, after a series of workers had developed hepatic angiosarcoma with an association to their exposure to vinyl chloride (VC) [Crech et al., 1974]. In the 1970s and 1980s, this study was followed by about 20 further case series and small epidemiologic studies, primarily focusing on the association of VC exposure and hepatic tumors [Kielhorn et al., 2000]. At the same time, there are several reports on the carcinogenicity of VC in rats and other rodents showing that VC is both genotoxic and carcinogenic, and causes a variety of tumors in various animals [Drew et al., 1983; Feron et al., 1981; Til et al., 1991; Viola et al., 1971; Viola, 2001]. VC is metabolically converted by microsomal monooxygenases in the liver into chloroethylen oxide, as the ultimate alkylating, mutagenic, and carcinogenic metabolite of VC, which reacts with DNA bases to yield etheno adducts [reviewed in Barbin, 1998]. The generation of etheno adducts and their reactions are extensively reviewed by Barbin [2000]. Etheno adducts (or ethenobases) generate mainly base-pair substitution mutations. In three out of six patients suffering from hepatic angiosarcoma with previous VC exposure, the typical A:T to T:A transversions in the codons 179, 249, and 255 have been described [Hollstein et al., 1994; Trivers et al., 1996]. These data are supported by the findings of *p53* mutations in 44% of rat hepatic angiosarcomas exposed to VC. Most of the point substitutions occurred at A:T base-pairs. In total, there were five A:T to T:A and two A:T to C:G transversions as well as two A:T to G:C and three G:C to A:T transitions [Barbin, 2000]. According to these results hotspots on the codons 203, 235, and 253 were suggested, while it has to be emphasized that the mutations at codon 253 in rats correspond to the mutations at the codon 255 found in human tumors [Hollstein et al., 1994]. In the same study, an A:T to T:A transversion has been seen in one of eight HCCs caused by the VC exposure.

The available data on the association of VC exposure and the development of HCC are less conclusive. The first report on HCC among two VC workers was published in 1983, followed by some epidemiological studies that suggested an association between VC exposure and HCC [Evans et al., 1983; Tamburro et al., 1984], although other possible risk factors of HCC such as alcohol consumption or chronic HBV infection were not excluded in these studies [Saurin et al., 1997]. One recent study

describes 11 out of 18 *p53* mutations in HCC from VC exposed patients, without any A:T to T:A transitions, which therefore, might be typically associated only with VC-induced hepatic angiosarcoma, but not with HCCs [Weihrauch et al., 2000]. Instead, CpG site mutations (five of 11) have been found on the hotspot codons 175, 248, and 273 that are also common in HCC due to alcohol consumption or HBV infection. Therefore, one possible conclusion is that VC may play a more indirect role in HCC, e.g., by triggering the cellular turnover based on direct cytotoxic or inflammatory effects [Weihrauch et al., 2000]. A:T to T:A or G:C to A:T transitions in the *Ha-ras* and *Ki-ras* genes are also found in human and rat hepatic angiosarcomas [Barbin, 2000; Marion and Boivin-Angele, 1999].

The above reported mutation spectra in liver tumors (angiosarcoma and HCC) that are associated with vinyl chloride exposure are clearly distinct from those seen in sporadic or other carcinogen-induced liver tumors.

### Oxyradical Overload Diseases

There are several studies linking oxidative stress and chronic inflammation with an increased cancer risk [Christen et al., 1999]. Inflammation leads to production of free radicals that can damage DNA (Fig. 3). Accordingly, several oxyradical overload diseases are associated with an increased risk of cancer, e.g., ulcerative colitis, pancreatitis, viral hepatitis, hemochromatosis, and WD. Hemochromatosis and WD are genetic disorders characterized by an excessive absorption, and an accumulation of iron and copper in various organs, but especially in hepatocytes. This leads to cirrhosis, and besides other complications, to 200-fold increased risk for the development of HCC in hemochromatosis, while in the case of WD, a lower incidence of HCC has been reported [Hussain et al., 2000]. This might be due to a shorter lifespan caused by hepatic failure or cardiac complications. Both diseases are based on germ-line mutations [Hussain et al., 2000]. In 1996, Britton described evidence of oxidative stress with a subsequent generation of reactive aldehydes in patients with these diseases [Britton, 1996]. Oxidative stress and the generation of reactive species may cause mutations in cancer-related genes, and affect key regulator proteins of DNA repair, cell cycle, and apoptosis [Laval et al., 1997; Mannick et al., 1999; van der Vliet et al., 1997; Zech et al., 1999].

The *p53* mutation load in nontumorous human liver tissue may be a biomarker of oxyradical damage and can help identify individuals with an increased risk of liver cancer. Therefore, we have determined the frequency of mutated *p53* alleles in nontumorous liver tissue from patients with hemochromatosis or

WD [Hussain et al., 2000]. In comparison to normal liver samples, we have found higher frequencies of G:C to T:A transversions at codon 249 ( $P < 0.001$ ) and C:T to A:T transversions as well as C:G to T:A transitions at codon 250 ( $P < 0.001$  and  $P < 0.005$ ) in liver tissue from 12 WD patients. In eight patients with hemochromatosis, we have found a higher frequency of G:C to T:A transversions at codon 249 ( $P < 0.05$ ). Interestingly, the inducible nitric oxide synthase (iNOS or NOS2) was elevated in 60% of the WD and 28% of the hemochromatosis patients in the liver, which suggests that nitric oxide is a source of increased oxidative stress.

Several studies support findings of an increased *p53* mutational load and its association with an increased level of oxyradical species. It has been shown, that excess iron and copper can produce Fenton oxidants, e.g., hydroxyl radicals ( $\text{OH}^\bullet$ ) [Carrier et al., 2001; Hussain et al., 1994]. G:C to T:A and C:G to A:T transversions at the *p53* codons 249 and 250, respectively, were found following the treatment of normal human fibroblasts with  $\text{H}_2\text{O}_2$  and  $\text{FeCl}_3$  [Ambs et al., 1999; Hussain et al., 1994]. Furthermore, based on previous reports showing evidence of lipid peroxidation as well as the subsequent generation of etheno adducts in both WD and hemochromatosis patients [Nair et al., 1998; Niemela et al., 1999; Sokol et al., 1994; Young et al., 1994], we exposed a normal lymphoblastoid cell line with wild-type *p53* to 4-hydroxynonenal, which is an unsaturated aldehyde involved in lipid peroxidation, and observed an increase in G:C to T:A transversions at *p53* codon 249. In addition, reactive oxygen species can activate signal transduction pathways resulting in the transcriptional induction of growth-competence-related oncogenes, e.g., *c-fos*, *c-jun*, and *c-myc* [Cerutti and Trump, 1991]. Taken together, these results are consistent with the hypothesis that the generation of oxygen and/or nitrogen species as well as etheno adducts from iron or copper overload in hemochromatosis or WD may cause *p53* mutations.

**HBV and HCV as Oncoviruses in Liver Carcinogenesis**

The hepatitis viruses, HBV and HCV, induce liver injury, hepatocyte death, and promote hepatocarcinogenesis. It is still not clear whether it is the virus infection causing the tumor initiation or whether it is the subsequent inflammation leading to liver regeneration and cirrhosis, which act as a tumor promoter in hepatocarcinogenesis. Available data suggest that viral proteins undergo various interactions with host proteins leading to an alteration of the cellular gene expression, which may contribute to a virus-associated carcinogenesis (Fig. 4).

About 40% of all HCCs worldwide occur in patients infected with HBV [reviewed in Hofseth et al., 2002]. HBV is a hepadna virus with a partially double-stranded DNA molecule, which is about 3.2 kilobases long and consists of four open reading frames encoding the envelope protein, the nucleocapsid (core) protein, the viral reverse transcriptase, and the HBx protein. HBx is required for the transcription of the viral genome. Among these, the HBx protein has been the focus of attention, because it seems to play a strong causal role in HCC.

Based on many earlier studies, it is now well established that most of the HCCs related to HBV infection contain HBV DNA sequences integrated into the host chromosomal DNA [Robinson, 1994]. This integration is highly variable and random [Galloway and McDougall, 1983]. In association with HBV infection, amplifications or single-base mutations of oncogenes are rare events in human HCCs [de The et al., 1987; Wang et al., 1990]. Instead, rearrangements at cellular DNA sites, where the HBV genome is integrated, result often in translocations [Hino et al., 1986; Meyer et al., 1992], inverted duplications [Hino et al., 1989; Tokino and Matsu- bara, 1991], deletions [Hino et al., 1986; Nakamura et al., 1988; Rogler et al., 1985], and possibly recombinations [Hino et al., 1991]. These

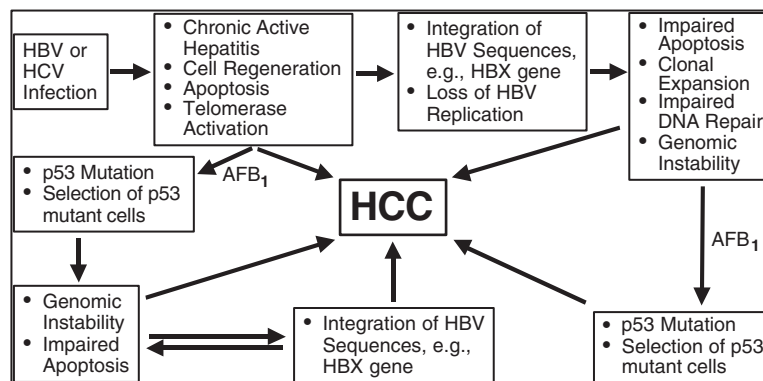


FIGURE 4. A model for viral-chemical interactions in multistage HCC (HBV, hepatitis B virus; HCV, hepatitis C virus; AFB<sub>1</sub>, aflatoxin B<sub>1</sub>).

HBV-induced chromosomal alterations may result in the loss of cellular regulatory genes, such as tumor suppressor genes, necessary for cell cycle control, differentiation, and apoptosis (Fig. 4).

Another effect of viral infection is the transactivation of cellular genes by the gene products of the integrated HBV DNA that might be involved in HCC. The most commonly integrated HBV gene is the HBx gene (Fig. 5) [Unsal et al., 1994]. In addition, it has been shown that only mammalian hepatitis viruses (e.g., HBV and woodchuck) are associated with HCC, while avian hepatitis viruses, which do not encode the HBx protein, did not [reviewed in Hofseth et al., 2002]. Therefore, recent studies have focused on the pathobiological effects of HBx. The HBx protein is a transcriptional coactivator of cellular and viral genes [Kim et al., 1991; Spandau and Lee, 1988; Twu and Schloemer, 1987]. For example, it alters the transcription through modulation of RNA polymerase II and III [reviewed in Hofseth et al., 2002]. In addition, it acts as a cotranscription factor, e.g., for the major histocompatibility complex (MHC) [Zhou et al., 1990], interleukin-8 [Mahe et al., 1991], epidermal growth factor receptor [Menzo et al., 1993], and oncogenes like *c-myc*, *c-jun/fos* [Natoli et al., 1994] or the *ras*-signalling [reviewed in Hofseth et al., 2002]. Several hypotheses have been proposed to explain how HBx mediates transactivation. HBx may interact with cellular transcription factors to influence gene expression. Alternatively, HBx can activate transcription by modulating of several signal transduction pathways. The HBx may also alter the protein nuclear export pathway to influence transcription [Forgues et al., 2001].

In addition, HBx decreases *p53* binding to XPB [Wang et al., 1994], which is both involved in nucleotide excision repair and in transcription as

a basic transcription factor [Schaeffer et al., 1993] and interacts with various other DNA repair associated proteins or enzymes, including DNA helicases [Jia et al., 1997; Qadri et al., 1996]. HBx itself has been shown to decrease nucleotide excision repair [Becker et al., 1998; Groisman et al., 1999; Jia et al., 1999]. These interferences of HBx with the cellular DNA repair system provide another mechanism how HBV contributes to liver carcinogenesis.

We and others have shown that HBx binds to *p53* and inactivates *p53*-dependent activities [Elmore et al., 1997; Feitelson et al., 1993; Ueda et al., 1995; Wang et al., 1994, 1995] including *p53* sequence-specific DNA-binding activity in vitro [Wang et al., 1994] and *p53*-mediated transcriptional activation in vivo [Wang et al., 1994], and represses *p53* transcription [Lee and Rho, 2000]. Moreover, HBx deregulates cell-cycle check point controls and blocks *p53*-mediated apoptosis [Bennett et al., 1995; Feitelson et al., 1993; Lucito and Schneider, 1992; Miura et al., 1997; Wang et al., 1995]. Interestingly, tumor-derived HBx mutants that lacked their transcriptional cotransactivation activity as well as pro-apoptotic activity [Huo et al., 2001] still retained their *p53*-binding functions and blocked *p53*-mediated apoptosis (Fig. 6) [Sirma et al., 1999]. Furthermore, by losing the pro-apoptotic ability, the mutant HBx enhanced the transforming ability of *ras* and *myc* [Tu et al., 2001]. The abrogation of *p53*-mediated apoptosis by HBx [also reviewed in Arbutnot et al., 2000; Bergsland, 2001; Jia et al., 1997; Murakami, 2001] may provide a selective clonal advantage for preneoplastic or neoplastic hepatocytes and contribute to hepatocellular carcinogenesis.

In an attempt to determine the role of HBx in the early pathogenesis of HCC, we utilized the NCI Oncochip microarray that contained 2,208 human cDNA clones to examine the gene expression profile in various in-vitro models as well as in liver samples from patients with chronic active hepatitis B. With the alterations of many cellular genes (Table 2), our findings are consistent with the hypothesis that these deregulations by oncogenic HBx may be an early event that favors hepatocyte proliferation during liver carcinogenesis [Wu et al., 2001]. Moreover, in a study from Wu et al. [2002], gene expression profiles from freshly isolated human primary hepatocytes infected with a replication-defective adenovirus containing HBx were analyzed by Serial Analysis of Gene Expression (SAGE). Interestingly, most of the HBx-upregulated transcripts could be clustered into three major classes, including genes that encode ribosomal proteins, transcription factors with zinc-finger motifs, and proteins associated with protein degradation pathway. These results suggest that HBx may function as a major disregulator in common cellular pathways that regulate protein synthesis, gene transcription, and protein degradation.

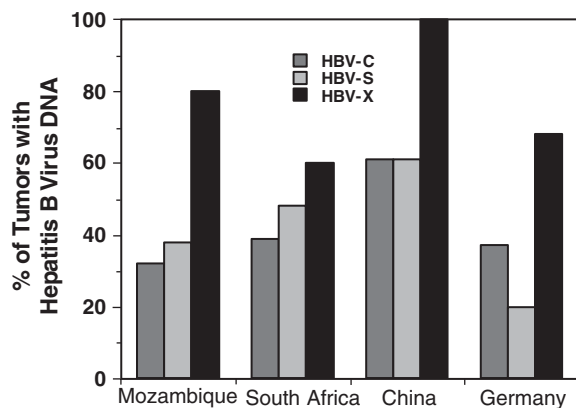


FIGURE 5. Integration of hepatitis B virus genes in human HCC from different geographic regions. In all tested areas, the HBV-X gene (black bars) is the most commonly integrated HBV-gene compared with the HBV-S gene (light grey bars) or the HBV-C gene (grey bars).

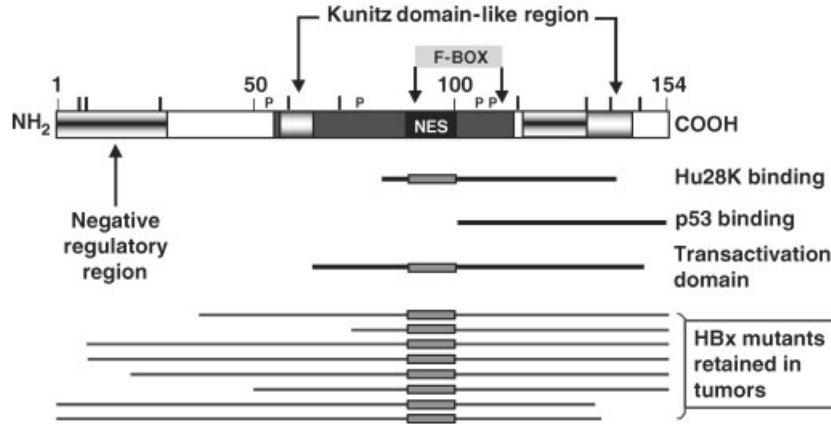


FIGURE 6. The HBV-X gene product and its mutations. Both missense mutations and deletions are frequent in tumor-derived HBV-X. The nuclear export signal (NES) and retention of p53 binding are found in the integrated HBV-X gene in human cancers.

TABLE 2. Examples of Genes Coregulated in Both Primary Human Hepatocytes Expressing HBx in vitro and Chronic Active Hepatitis B Infection in vivo [Wu et al, 2001], Differentially Expressed by 2-fold or More

Functional class	Upregulated	Downregulated
Tumor suppressors		<i>p53</i> <i>APC</i>
Cell cycle regulators, Growth factors/ receptors	<i>Cyclin D3</i> <i>Cdk4</i>	<i>TGF-β</i> <i>p21<sup>waf1</sup></i>
Cytokines and their receptors Apoptosis	<i>IGF-1R</i> <i>Interferon α/β</i> <i>receptor 2</i> <i>Bcl-XL</i> <i>Fas</i> <i>Caspase 4</i>	<i>gas6</i> <i>IL6</i>

When compared with HBV infection, the hepatitis virus C (HCV) infection has different characteristics and outcomes [Shiratori et al., 1995]. HCV is a 9.6 Kb single-stranded RNA flavivirus causing more severe liver inflammation when compared with HBV infection as more patients become cirrhotic (70% vs. 50%) and more patients with HCV-associated cirrhosis develop HCC when compared with HBV-associated cirrhosis cases (75% vs. 29%) [Ikeda et al., 1993]. The HCV genome is not integrated into the host genome and consists of one core protein, two envelope proteins, E1 and E2, and several nonstructural proteins (NS2-5) functioning in viral replication. One study has shown that the core protein of HCV induces HCC in transgenic mice, suggesting a direct involvement of the core protein in HCC [Moriya et al., 1998]. The HCV core protein interacts with several intracellular pathways [Aoki et al., 2000; Kato et al., 2000], e.g., indirect activation of the *Raf-1* kinase, tumor necrosis factor-related receptor (LT-βR), and of NF-κB pathway activation [You et al., 1999], leading

to inhibition of TNF-α-induced and *Fas*-mediated apoptosis [Marusawa et al., 1999].

However, depending on the cellular background there are also studies showing contradictory effects [Zhu et al., 1998]. There also exist contradictory data for the studies showing that the HCV core protein is capable of suppressing the transcriptional activity of the *p53* promoter by using different cell lines [Lu et al., 1999; Ray et al., 1997]. Two recent studies have tried to provide a better insight into the pathogenesis of HCV-related HCC by using the microarray approach [Honda et al., 2001; Shackel et al., 2002]. Both studies show an upregulation of pro-inflammatory, pro-proliferative, and pro-apoptotic genes in HCV cirrhosis, and speculate that such genes may reflect the underlying mechanisms of the propensity of HCV-associated cirrhosis to develop HCC [Honda et al., 2001; Shackel et al., 2002]. However, these and particularly the contrasting results, illustrate the need of further research on HCV infection and its etiological significance in HCC.

### Oxyradicals in Liver Carcinogenesis

Growing evidence indicates that nitric oxide (NO•), an important bioregulatory and signalling molecule, may play a significant role in carcinogenesis [Ambs et al., 1997; Bredt and Snyder, 1994; Hentze and Kuhn, 1996; Moncada et al., 1991; Nathan and Xie, 1994; Tamir and Tannenbaum, 1996]. NO• is catalyzed by a family of enzymes known as nitric oxide synthases (NOS) [Forstermann and Kleinert, 1995; Marletta, 1993]. The isoforms NOS1 and NOS3 are found to be constitutively expressed, e.g., in neurons (NOS1) or endothelial cells (NOS3). They produce NO• levels ranging from pico to nano molar concentrations. In contrast, NOS2 (also called iNOS) requires generally induction, but is able to produce

NO<sup>•</sup> concentrations in the micromolar range [reviewed in Ambs et al., 1997]. NOS2 gene expression can be induced by either bacterial endotoxins or proinflammatory cytokines [Lombard and Guarente, 2000; Nussler et al., 1992; Wild et al., 1986] in many cell types including macrophages [Xie et al., 1992] and hepatocytes [Lombard and Guarente, 2000; Mowat et al., 1990] as well as in a variety of human tumors [reviewed in Ambs et al., 1997]. During chronic viral hepatitis the upregulation of certain proinflammatory cytokines, like TNF- $\alpha$  and IFN- $\gamma$ , has been repeatedly demonstrated [Gonzalez-Amaro et al., 1994; Mihm et al., 1996]. These proinflammatory cytokines, TNF- $\alpha$ , IFN- $\gamma$  and IL-1, induce NOS2 gene expression, leading to increased NO<sup>•</sup> concentrations in human hepatocytes [de Vera et al., 1996; Laskin et al., 1998]. In addition, NOS2 is also induced directly by the hepatitis B and C viruses. In-vitro studies from Elmore et al. [1997], Amaro et al. [1999], and Majano et al. [1998] demonstrate that the HBx protein is capable of transcriptional transactivation of NOS2. An induction of the NOS2 expression has also been seen after hepatocytes are exposed to woodchuck hepatitis virus surface antigen (W<sub>h</sub>sAg) [Liu et al., 1994]. In patients with HCV infection, a consistent upregulation of hepatic NOS2 has been shown [Kane et al., 1997]. The biological effects caused by NO<sup>•</sup> are multiple and complex, and depend on the site of production, target cells, local concentration, presence of metals, etc. [reviewed in Ambs et al., 1997; Taylor et al., 1998]. Besides its physiological functions, NO<sup>•</sup> can be mutagenic and angiogenic by mediating tumor vascularization and blood flow. High concentrations of NO<sup>•</sup> can cause DNA damage, either directly or through secondary molecules, by nitrosative deamination, DNA strand breakage, and DNA modifications, e.g., nitration, by N-nitrosamines or peroxy-nitrate (Fig. 3) [reviewed in Ambs et al., 1997]. The latter one may also cause the formation of DNA-reactive lipid-peroxidation intermediates [Ambs et al., 1997]. In this context, DNA-repair proteins play a major role in preventing such oxidative stress-related DNA mutations, but many of these enzymes are themselves vulnerable to oxidative stress [reviewed in Ambs et al., 1997]. There are several studies showing the mutagenic effect of high NO<sup>•</sup> levels [Tretyakova et al., 2000; Zhuang et al., 1998; Zhuang et al., 2000]. One good example demonstrating the relevance of oxidative stress in chronic viral hepatitis is the increased level of 8-hydroxydeoxyguanosine, which is a modified DNA base product by reactive oxygen species capable of inducing G:C to T:A transversion and it is a recognized marker for oxidative DNA damage [Shimoda et al., 1994].

We and others were able to show that this NO<sup>•</sup>-related DNA damage leads to p53 accumulation and p53-mediated apoptosis [Forrester et al., 1996; Mess-

mer and Brune, 1996]. Moreover, we presented results indicating that p53 is a transcriptional transrepressor of NOS2 expression in vivo and attenuates an excessive NO<sup>•</sup> production in a regulatory negative feedback loop [Ambs et al., 1998a; Forrester et al., 1996; Hussain et al., 2001]. Taken together, NO<sup>•</sup> production could contribute to human cancer progression by selecting against wild-type p53 and selecting for mutant p53 cells. In fact, we have found a significant association as well as a dose-response relationship between p53 mutations (G:C to A:T transition at CpG sites) and an increased NOS2 activity in patients with colon cancer [Ambs et al., 1999]. Furthermore, we and others have demonstrated a positive association of NOS2 expression and a comparable p53 mutational spectrum in lung cancer [Ambs et al., 1998b; Fujimoto et al., 1998]. This p53 mutation spectrum is consistent with increased rates of N<sub>2</sub>O<sub>3</sub>-induced deamination of 5-methylation at CpG sites.

### MODEL OF LIVER CARCINOGENESIS

HCC is an aggressive malignancy with a poor prognosis. While the pathogenesis of HCC is multifactorial and develops over several stages, a number of etiological factors have been identified and their mechanistic role in hepatocarcinogenesis is currently under investigation.

Epidemiological studies clearly have shown that infection with the HBV or/and HCV as well as the increased dietary intake of AFB<sub>1</sub>, are major risk factors for the development of HCC. Because of these risk factors, HCC is the predominant cause of cancer mortality in southern China and sub-Saharan Africa. There is a clear molecular link between AFB<sub>1</sub> exposure leading to typical G:C to T:A transversions at the third base of p53 codon 249 and liver cancer. HBV infection leads to a random integration of viral DNA sequences, resulting in frequent chromosomal alterations that may contribute to loss of heterozygosity or loss of tumor suppressor genes as commonly found for the short arm of chromosome 17, which includes the p53 tumor suppressor gene. p53 function may also be inactivated by the physical interactions with the HBx protein that acts as a transcriptional transactivator on various genes and DNA-repair proteins. Compared with HBV infections, patients suffering from HCV infection develop more severe inflammation, more often cirrhosis, and finally HCC. With contradictory data according to the cellular background, the HCV core protein is capable of inhibiting the p53 promoter and interacts with various intracellular pathways that are, e.g., involved in resistance to Fas-mediated apoptosis. Both hepatitis viruses increase NOS2 gene expression and activation of the protecting p53 response pathway to cellular stress. Considering the large population of cells at risk, rarely p53 mutations are induced and clonal growth of

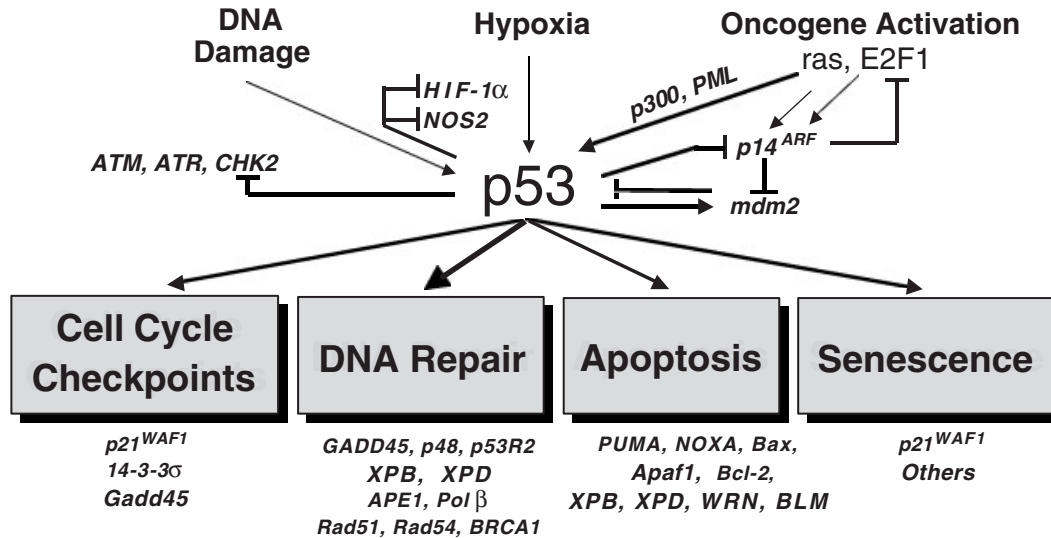


FIGURE 7. Schematic showing *p53* at the crossroads of cellular stress response pathways. Negative feedback loops both upstream and downstream of *p53*, precisely regulate *p53* function. Examples of some of the proteins mediating *p53* functions are listed.

*p53* mutant cells escape the *p53*-mediated cell cycle checkpoints, DNA repair mechanisms, or apoptosis (Fig. 7).

Oxidative/nitrosative stress, mediated by NO, which is also induced by the cytokine profile of various inflammatory hepatic disorders like HBV and HCV infection, WD, or hemochromatosis, can act as an endogenous carcinogen and provides a clonal growth advantage for cells with *p53* mutations.

Although our understanding of the pathophysiological mechanisms involved in hepatocarcinogenesis has clearly improved and resulted in more efficient preventive measures in the recent years, our HCC therapeutic options are still limited.

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