MINIREVIEW

Genetic and Epigenetic Control of Molecular Alterations in Hepatocellular Carcinoma

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Comparative analysis of hepatocellular carcinoma (HCC) in rat strains that are either susceptible or resistant to the induction of HCC has allowed the mapping of genes responsible for inherited predisposition to HCC. These studies show that the activity of several low penetrance genes and a predominant susceptibility gene regulate the development of hepatocarcinogenesis in rodents. These studies shed light on the epidemiology of human HCC. The identified genes regulate resistance to hepatocarcinogenesis by affecting the capacity of the initiated cells to grow autonomously and to progress to HCC. Analysis of the molecular alterations showed highest iNos cross-talk with IKK/NF-kB and RAS/ERK pathways in most aggressive liver lesions represented by HCC in the susceptible F344 rats. Unrestrained extracellular signal-regulated kinase (Erk) activity linked to proteasomal degradation of dual-specificity phosphatase 1 (Dusp1), a specific ERK inhibitor, by the CKS1-SKP2 ubiquitin ligase complex was highest in more aggressive HCC of genetically susceptible rats. Furthermore, deregulation of G1 and S phases of the cell cycle occurs in HCC of susceptible F344 rats, leading to pRb hyperphosphorylation and elevated DNA synthesis, whereas a block to G1-S transition is present in the HCC of resistant BN rats. Importantly, similar alterations in the signaling pathways that regulate cell cycle progression were found in human HCC with poorer prognosis (as defend by patients' survival length), whereas human HCC with better prognosis had molecular characteristics similar to the lesions in the HCC of resistant rat strains. This review discusses the role of molecular alterations involved in the acquisition of resistance or susceptibility to HCC and the importance of genetically susceptible and resistant rat models for the identification of prognostic markers, and chemopreventive or therapeutic targets for the biological network therapy of human disease. Exp Biol Med 234:726–736, 2009

Key words: hepatocarcinogenesis; genetic predisposition; signaling pathways; cell cycle

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent human cancers, with ~1 million of newly diagnosed cases each year. Highest frequencies occur in sub-Saharan Africa and far eastern Asia, where hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are endemic, and in geographic areas where food contaminated with Aflatoxin B1 is consumed (1–3). HCC incidence is rising, even in countries with relatively low incidence, mainly due to an increased incidence of alcoholic cirrhosis and type 2 diabetes (4). HCC is a rapidly fatal disease, partial liver resection or liver transplantation is potentially curative, but only minorities of cases are amenable to these treatments.

Human HCC generally develops in livers with chronic disease such as hepatitis, cirrhosis, hemochromatosis, etc. Recognizable early lesions, probably arising from hepatic progenitor cells (5), are represented by different subtypes of foci of altered hepatocytes (FAH; Fig. 1). Dysplastic (adenomatous, neoplastic) nodules, larger than a liver lobule, are considered evolution products of FAH and are regarded as premalignant lesions (6). Dysplastic nodules are generally composed of small cells showing mild trabecular disarrays, increased nuclear-cytoplasmic ratio, and tendency to cytoplasmic basophilia.

In hepatocarcinogenesis models, irreversibly initiated cells can undergo clonal expansion in response to the administration of promoting agents for several weeks (7).

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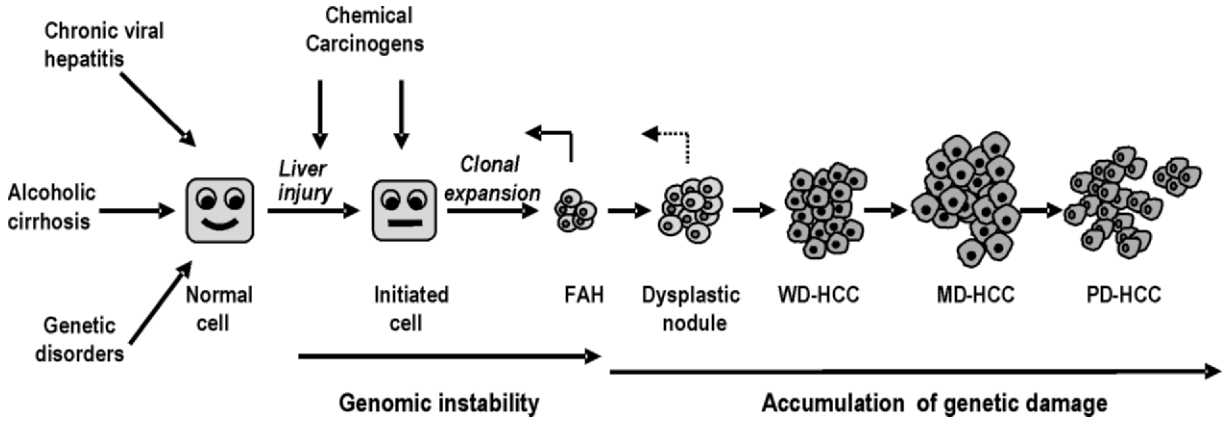

Figure 1. Schematic representation of liver carcinogenesis. Persistent liver injury due to oxidative DNA damage and chemical carcinogens induces genomic instability and initiates liver cell malignant transformation. Clonal expansion of initiated cells leads to the development of foci of altered hepatocytes (FAH), some of which can undergo phenotypic reversion (remodeling; reverse arrows). Accumulation of genetic damage leads to the development of autonomously growing dysplastic nodules which progress to well differentiated (WD), moderately differentiated (MD) and poorly differentiated (PD) invasive hepatocellular carcinomas (HCC).

Different clones of initiated cells evolve to a heterogeneous population of FAH and, further, to early tumor nodules. These lesions, similar to nodules developing during human liver carcinogenesis, express specific patterns of marker genes, controlling carbohydrate and xenobiotics metabolism, some cellular structural components, and DNA synthesis (7, 8). About 80% of the initiated cells express, in rats, glutathione S-transferase (GST) 7–7. Numerous premalignant lesions acquire the capacity to growth autonomously, further evolve to neoplastic nodules, and then to well differentiated carcinomas which progress to moderately and poorly differentiated HCCs (Fig. 1; Ref. 8). When the treatment with tumor promoters is suspended before the appearance of neoplastic lesions, many premalignant liver lesions do not further evolve or disappear by a process named "remodeling" (7), characterized by apoptosis mediated cell death or re-differentiation of preneoplastic lesions.

The development of cancers in mammals depends on the accumulation within somatic cells, of a number of genetic alterations including activation of proto-oncogenes and inactivation of tumor suppressor genes. Genomic instability, fueled by DNA damage and errors in DNA repair, plays an important role in the accumulation of these alterations (1–3, 9). Several common morphologic, biochemical and biologic factors have been found between human and rodent preneoplastic and neoplastic liver lesions (2, 3, 9, 10). This indicates that, independently of the etiological factors, roughly the same classes of genetic targets are involved in the genesis of the lesions, and suggests that the basic mechanisms of HCC development are similar in different species.

The frequency of HCC shows great variations amongst different human populations exposed to the same environmental risk factors. This suggests that additional environmental and/or genetic factors may be involved in the pathogenesis of the disease. Polymorphisms in several genes, including those encoding cytochromes P450 2E1 and 2D6, aldehyde dehydrogenase, arylamine N-acetyltransferase 2, epoxide hydrolase, L-*MYC*, and glutathione S-transferase, have been associated with increased risk of HCC (10). Studies on families at risk and segregation studies on human population support the existence of a genetic predisposition to hepatocarcinogenesis (11). This review shortly focuses on the polygenic control of the susceptibility to liver cancer and illustrates recent findings on the influence of susceptibility genes on HCC development and prognosis through the control of genetic and epigenetic mechanisms of hepatocarcinogenesis.

Genetic Susceptibility to Hepatocarcinogenesis

Susceptibility to the development of tumors in rodent and human tissues has been attributed to interindividual variations in the inducibility of some cytochrome P-450 isoforms that are able to activate carcinogens (10). However, various rat strains resistant to hepatocarcinogenesis, including NAR, ODS and some Wistar strains, exhibit a higher inducibility of cytochrome P-450 isoforms, by phenobarbital and 3-methylcholanthrene, than susceptible strains including Fisher 344 (F344) (10). Furthermore, a body of evidence indicates that susceptibility genes do not influence the initiation of hepatocarcinogenesis in rodents, but affect the capacity of preneoplastic lesions to grow autonomously and evolve to full malignancy (10, 12).

The analysis of genetic susceptibility to liver cancer has led to the mapping of different *h*epato*c*arcinogenesis *s*usceptibility and *r*esistance (*Hcs, Hcr*) loci in mice and rats, and various *l*iver *n*eoplastic *n*odule *r*emodeling (*Lnnr*) loci in rats (10). These studies suggest the involvement of genetic factors in determining susceptibility to hepatocarcinogenesis. These factors include different subsets of low-penetrance genes, and a gene with higher penetrance, in different subsets of population. This model is in keeping with human HCC epidemiology (11). Mouse *Hcs1–Hcs6*

Table 1. Loci Linked to Hepatocarcinogenesis in Mouse and Rat

Locus	Chrom.	Strain[a]	Ref.
		Mouse	
Hcs1, Hcs2, Hcs3	7, 8, 12	(*C3H/HeJ × A/J)F2	13
Hcs4, Hcs5, Hcs6	2, 5, 19	(*C3H/HeJ × Mus Spretus)F1 × C57BL/6J[1]	13
Hcs7	1	(*C3H/HeJ × C57BL/6J) × C57BL/6J[2]	14
Hcf1	17	(C57BL/6J × C57BR/cdj) × C57BL/6J[3] (C57BL/6J × C57BR/cdj) × C57BR/6J[3]	15
Hcf2	1	(C57BL/6J × c57BR/cdj)F2	15
Hcr1, Hcr2	4, 10	(*DBA2J × C57BL/B6)	16
Hpcr3		(*C3H/HeJ × BALB/c)F2	17
		Rat	
Rcc	20p12	ACP (inbred), R21, R33 (congenics)	18
Hcs1, Hcs2	7, 1	(*F344 × BN) × F344	19
Hcr1, Hcr2, Hcr3	10, 4, 8	(*F344 × BN) × F344	19
Hcs3, Hcs4	1, 16,	(*F344 × BN)F2	20
Hcr9, Hcr10, Hcr11, Hcr12	4, 8, 6, 3	(*F344 × BN)F2	20
Hcs5, Hcs6, Hcs7	1, 14, 15	(*F344 × COP)F2	21
Hcr13, Hcr14	4, 18	(*F344 × COP)F2	21
Lnnr1, Lnnr2	7, 1	(*F344 × BN)F2	10
Lnnr3, Lnnr4, Lnnr5, Lnnr6	2, 13	(*F344 × COP)F2	21
F344.BN-*(D16Nit5–D16Rat65)*	16	Recombinant congenic	24
Drh1a/b/c	1	(*F344 × DRH)F2	22
Drh2a/b	4	(*F344 × DRH)F2	22
DRH.F344-*(D1Mgh8–D1Mgh12)*/Kyo	1	Recombinant congenic	23

[a] The asterisks indicate the susceptible strains.

quantitative trait loci (QTLs) were identified on chromosomes 7, 8, 12, 2, 5, and 19, respectively, in urethane-treated hybrid mice generated by crossing susceptible and resistant strains of mice (Ref. 13; Table 1). Recombinant congenic B6.C3H(*D1Mit5–D1Mit17*) and B6.BR(*D1Mit5–D1Mit17*) strains, generated by introgressing a ~70 cM segment (between *D1Mit5* and *D1Mit17*) from C3H or C57BR/cdj (Br) susceptible mice, onto a B6 background, allowed the identification of a potent *Hcs7* locus, sufficient to confer susceptibility to liver cancer, in the distal arm of chromosome 1 (14). Two loci, denominated *Hcf1* and *Hcf2* (*h*epatocarcinogenesis in *f*emales), have also been identified in crosses between BR and B6 mice (15). *Hcf1* and to a lower extent *Hcf2* accounted for the higher sensitivity of male and female BR mice to hepatocarcinogenesis.

In addition to susceptibility loci, two resistance loci, *Hcr1* and *Hcr2,* with negative phenotypic effects have been discovered on mouse chromosomes 4 and 10, respectively (Ref. 16; Table 1). Resistant F1 mice were generated by crossing the resistant BXD-15 recombinant inbred strain, carrying *Hcr* genes, to the susceptible recombinant BXD-11 strain, strongly suggesting that *Hcr* genes may modify the activity of sensitivity loci (16).

The genome of the BALB/c mouse strain provides alleles that semi-dominantly inhibit development of HCC in (Balbc x C3H/He)F1 susceptible mice (13). Recent genome-wide linkage analysis in a F2 population produced by crossing the BALB/c to the C3H/He mouse strain revealed a hepatocarcinogen resistance 3 (*Hpcr3*) locus with a major role in the resistance to urethane-induced hepatocarcinogenesis (17). A gene expression profile of normal adult mouse liver showed that the E2F1 pathway modulates the susceptibility of BALB/c, C3H/He, and F1 mice to hepatocarcinogenesis.

The first locus regulating the susceptibility of rats to chemical hepatocarcinogenesis, denominated *rcc* locus, has been identified at the telomeric end of chromosome 20 of MHC-recombinant rat strains, congenic for the MHC genes and its linked region *grc* (growth reproduction complex) (18). This locus has many properties in common with tumor-suppressor genes: it is recessive, its deletion causes phenotypic susceptibility to various carcinogens, and it inhibits tumor development in many organs and tissues, including liver, skin, kidney and mesenchyma.

Recently, the Brown Norway (BN) and Copenhagen (Cop) rat strains, strongly resistant to hepatocarcinogenesis (10), have been largely used to map the genes determining a phenotype susceptible to HCC. These strains, crossed to the susceptible F344 rats, dominantly transmit their resistance to (BN x F344)F1 (BFF1) and (Cop x F344)F1 (CFF1) rats. Numerous loci have been identified by linkage analysis of male backcrosses and intercrosses of resistant BN and/or Cop rats to susceptible F344 rats (Table 1). They include rat *Hcs1* and *Hcs2* loci identified in BN × BFF1 backcross progeny (19), *Hcs3* and *Hcs4* loci identified in BFF2 rats (20), and *Hcs3*, *Hcs5*, *Hcs6* and *Hcs7* identified in CFF2 intercrosses (21). *Hcr* loci numbered 1 to 3 have been mapped to chromosomes 10, 4, and 8, respectively, in BN x BFF1 backcrosses (19). Four additional *Hcr* loci, numbered

from 9 to 12, (Rat genome database, www.rgd.mcw.edu/; previously numbered from 4 to 7) were identified on chromosomes 4, 8, and 6 of BFF2 rats (20). *Hcr13* and *Hcr14* (RGD; previously numbered 8 and 9) were mapped to chromosomes 4 and 18 of CFF2 rats (21). Moreover, the scanning of BFF2 and CFF2 rats genome led to the identification of *Lnnr1* and *Lnnr2* foci in BFF2, and of *Lnnr3, Lnnr4* and *Lnnr5* foci in CFF2 rats (10, 21). The functional effects of *Lnnr* loci are consistent with a model of hepatocarcinogenesis in which only a relatively small subset of early preneoplastic lesions is genetically programmed to evolve to HCC, whereas the remainder undergo phenotypic reversion. The importance of this phenomenon is underlined by the observation of hepatocarcinogenesis prevention by compounds inducing remodeling (9). Two clusters of genes designated collectively as *Drh1* and *Drh2,* and considered resistance genes, have been identified on chromosomes 1 and 4 in intercrosses between the resistant mutant DRH and F344 rats (Ref. 22; Table 1). Notably, on the basis of the chromosomal localization, *Dhr2* seems to correspond to *Hcr2* on chromosome 4, while *Drh1* corresponds to *Hcs3* and *Hcs5*, discovered in crosses between BN and F344 rats (19, 20).

The phenotypic effect of *Hcs3* locus in BFF2 rats, consisting of a marked increase in the volume of neoplastic nodules, accounts for 49% of the total phenotypic traits (10). In DRH rats, the presence of *F* alleles at the *Drh1* locus has a dominant positive effect on the number of FAH (about 100% increases) (22). These effects of *Hcs3*/*Drh1* locus have been recently confirmed in a congenic DRH.F344-*Drh1* strain in which an approximately 43 cM segment of *Drh1* from F344 rats has been introgressed onto a DRH background (23). These observations, considered altogether, are consistent with a major role for *Hcs3* in the predisposition to liver cancer.

Recently, we generated a congenic rat line by transferring the Hcs4 BN allele onto a F344 genetic background (24). By marker assisted selection of crosses, we narrowed the *Hcs4* locus to a 4.41 cM segment, including the LOD score peak at 9.04 cM from the centromere. This recombinant congenic strain, designated as F344.BN-*Hcs4*, showed a highly susceptible phenotype in the male recombinants, while the phenotypic behaviour of female rats closely corresponded to that of female BN rats, much more resistant to hepatocarcinogenesis than female F344 rats. The gonadectomy of recombinant rats induced a consistent decrease in the susceptibility of males and increased the susceptibility of the female. This behaviour suggests the existence in the F344.BN-*Hcs4* strain of hormone responsive resistance alleles that are contributed by the BN strain. These alleles are responsible for the resistance to hepatocarcinogenesis of female rats, an activity that is suppressed by male sex hormones and/or enhanced by female sex hormones. These observations indicate the presence on chromosome 16 of at least one gene that confers resistance to hepatocarcinogenesis to female rats. This represents a first demonstration of modulation by sex hormones of modifier genes controlling the predisposition to liver carcinogenesis.

The QTLs implicated in rodent hepatocarcinogenesis may include tumor modifier genes, genes targeted by modifiers, and genes not involved in the genetic susceptibility to cancer. A number of oncogenes, oncosuppressor genes, growth factors and growth factor receptor genes, genes involved in cell death and cell survival pathways, and DNA repair genes are positioned in these loci (10). Most of these genes play a role in liver tumorigenesis both in humans and rodents. However, although they are plausible candidates for genes involved in the determination of a susceptible or resistant phenotype, their association with the predisposition to liver cancer has not been demonstrated, as yet. Interestingly, some of the genes located in rodent QTLs correspond to human genes exhibiting frequent polymorphism such as *TNF-a, ESR1, MYC*, and genes encoding cytochrome P-450 isoforms (10). This suggests possible locations of some QTLs in the human genome. Unfortunately, there is no unambiguous evidence supporting the contribution of these genetic variants of candidate genes to the predisposition to human HCC. Systematic, comparative studies are needed to evaluate the role of the loci linked to hepatocarcinogenesis in the determination of cancer risk. The identification of genes whose activity is modulated by tumor modifiers would be of prime importance since it may reveal the functional pathways affected by modifiers, elucidate novel mechanisms of carcinogenesis and provide unique drug discovery opportunities for chemoprevention and therapeutic strategies.

Epistatic Interactions of Susceptibility/Resistance Genes with the Signaling Pathways

It is widely accepted that the interaction of DNA with carcinogens and reactive oxygen species (ROS), generated during carcinogen metabolism and inflammation accompanying early stages of hepatocarcinogenesis, results in genomic instability which renders the genome prone to the accumulation of severe DNA defects during the clonal expansion of initiated cells (25). 8-hydroxy-2′-deoxyguanosine is the major product of oxidative DNA damage which mispairs with adenine during DNA replication, resulting in GC → TA transversion. (26). Liver infiltration by phagocytes, during liver injury, provides a source of ROS which cause damage to DNA, proteins and lipids when their generation exceeds the ability of the antioxidant systems to remove them. Mounting evidence indicates a carcinogenic role for excessive production of nitric oxide ($NO^{\bullet}$) by inflammatory and cancer cells, as well as the interference of $NO^{\bullet}$ with signal transduction pathways involved in cancer cell growth (27, 28).

Aberrant iNOS Signaling in Hepatocarcinogenesis

Overproduction of inflammatory cytokines and growth factors during early stages of hepatocarcinogenesis dereg-

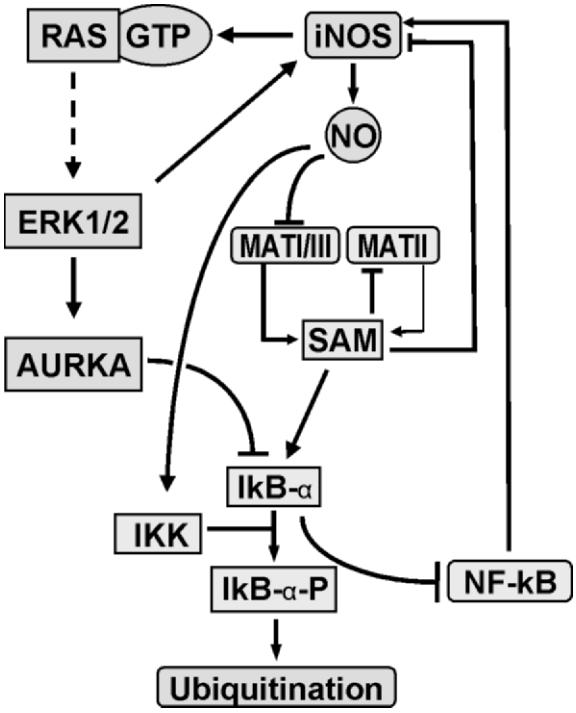

Figure 2. Schematic representation of the inducible nitric oxide synthase (iNOS) interplay with IKK/NF-kB and Ha-RAS/ERK signaling cascades. iNOS activates IkB kinase (IKK), which allows phosphorylation and proteasomal degradation of the nuclear factor kB (NF-kB) inhibitor, IkB-α, resulting in NF-kB activation. iNOS also activates H-RAS, thus triggering the mitogen activated protein kinase (MAPK) cascade which leads to extracellular signal-regulated kinase (ERK) activation. The activation of NF-kB by active ERK may occur through AURKA, which inhibits IkB-α. NO affects SAM synthesis by directly inhibiting the liver-specific methyl-thioadenosyl transferase I/III (MATI/III) isozymes. The fall in SAM levels cannot be fully compensated by an increase in the extrahepatic isozyme MATII, since this enzyme is inhibited by its reaction products. Inhibition of iNOS and activation of IkB-α by SAM may result in NF-kB inhibition. In contrast, iNOS activation by NF-kB and, possibly, by ERK1/2 enhances the cross-talk of iNOS with IKK/NF-kB and Ha-RAS/ERK signaling. Pointed and blunt arrows indicate activation and inhibition, respectively. The dotted arrow indicates a multistep process.

ulates the inducible nitric oxide synthase (*iNOS*), IkB kinase (*IKK*), and nuclear factor kB (*NF-kB*) axis (Fig. 2) (29). $NO^{\bullet}$ stimulates the expression of IKK protein, which phosphorylates the inhibitor of kappa B (IkB-α), thus allowing its ubiquitination and proteasomal degradation (28). As a consequence, the NF-kB family members may accumulate in the cytoplasm and reach the nucleus where they transactivate several growth-related genes, including c-*MYC,* cyclin and antiapoptotic genes (i.e. *BCLxL*), as well as inflammation-related nitric oxide synthetase family genes. Rise in $NO^{\bullet}$ production also stimulates angiogenesis by activating the hypoxia inducible factor-1 (*HIF-l*α) gene and its target, the vascular endothelial growth factor-α (*VEGF*-α) (30). Furthermore, as a modifier of proteins regulating cell function, $NO^{\bullet}$ can influence several downstream events in carcinogenesis, including cell-cycle checkpoints, apoptosis and DNA repair (30).

Recent work in our laboratory showed that *iNos*, *Ikk*, and *NF-kB* overexpression, in preneoplastic and neoplastic lesions induced by chemicals in rat liver, is associated with deregulation of IkB-α, decrease in IkB-α/NF-kB complex, and rise in NF-kB binding to DNA (27, 28). This indicates that the deregulation of the *iNos*, *Ikk*, and *NF-kB* axis is an early event during hepatocarcinogenesis, under control of susceptibility/resistance genes. Moreover, we found that i*Nos* induction in liver lesions of rats and c-Myc and c-Myc-TGF-α transgenic mice, reaches always higher levels in the most aggressive models represented by HCC of rats genetically susceptible to hepatocarcinogenesis and c-Myc-TGF-α transgenic mice. Remarkably, iNOS, IKK/NF-kB, and RAS/extracellular signal-regulated kinase (ERK) upregulation is highest in human HCC with poorer prognosis (as defined by patients' survival time, HCCP) and positively correlates with tumor proliferation, genomic instability, and microvessel density, and negatively correlates with apoptosis. Suppression of iNOS signaling by aminoguanidine leads to decreased growth and NF-kB and RAS/ERK expression, and increased apoptosis in HCC of c-Myc-TGF-α transgenic mice and human HCC cell lines (28). Conversely, block of NF-kB signaling by sulfasalazine or siRNA, or ERK signaling by UO126, causes iNOS downregulation in HCC cell lines, indicating that both NF-kB and ERK1/2 target *iNOS*. Therefore, the stimulation of NF-kB expression by *iNOS*, in preneoplastic and neoplastic liver, may establish a feedback loop leading to NF-kB accumulation (Fig. 2). Furthermore, these findings indicate that iNOS crosstalk with NF-kB and Ha-RAS/ERK cascades influences HCC growth and prognosis, suggesting that key components of iNOS signaling could represent important therapeutic targets for human HCC.

The Mitogen Activated Protein Kinase (MAPK) Cascade

The MAPK cascade is a critical signaling pathway supporting the proliferation of preneoplastic and neoplastic liver lesions (31). It transduces signals from tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), platelet derived growth factor receptor (PDGFR), hepatocyte growth factor receptor (HGFR/MET), and vascular endothelial growth factor receptor (VEGFR), bound to their ligands. In this cascade, active RAS (GTP-RAS) triggers the sequential activation of murine leukemia viral oncogene homolog 1 (RAF1), mitogen-activated protein kinase 1/2 (MEK1/2), and ERK1/2 (Fig. 3). Active ERK1/2 transactivates numerous growth-related genes, including *c-JUN, C-FOS, C-MYC,* and *ETS* (31). Most genes related to the MAPK cascade, such as c-Ha-*ras* and c-Ki-*ras*, c-*Raf*, c-*Fos*, and c-*Jun,* are overexpressed in FAHs, nodules, and HCCs induced in rodents (2, 3, 10). MAPK subfamilies described in mammals include ERK, c-JUN N-terminal kinases (JNKS) and p38α, β, γ, δ kinases (31, 32). The p38MAPK pathway is implicated in tumorigenesis. Liver-specific

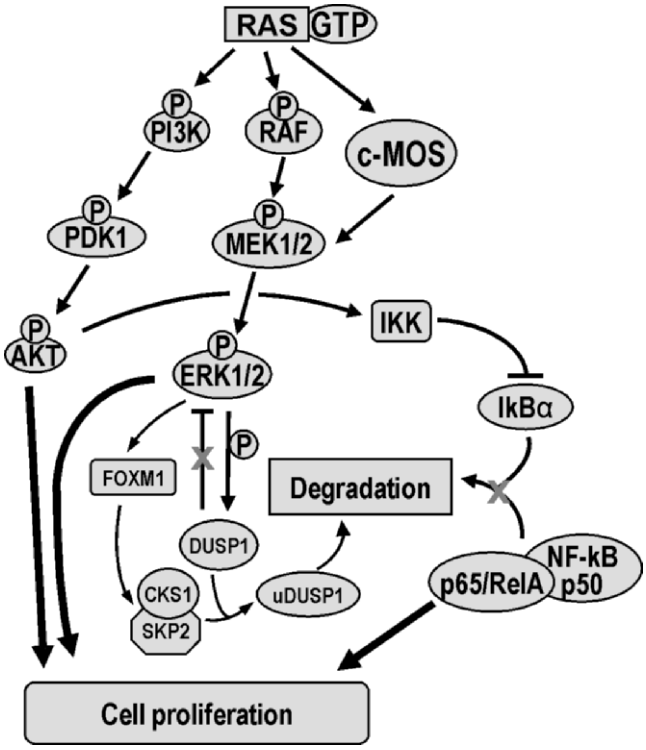

Figure 3. Schematic representation of RAS-GTP activated pathways leading to cell growth or apoptosis. The mitogen activated protein kinase (MAPK) cascade leads to ERK1/2 activation. Active ERK can down-regulate DUSP1 by phosphorylation at ser296 allowing the formation of the SKP2/CSK1/DUSP1 complex, which facilitates DUSP1 ubiquitination and proteasomal degradation. In addition, ERK may further contribute to DUSP1 proteolysis via induction of its target FOXM1, finally leading to transcriptional activation of SKP2 and CKS1 genes. These mechanisms together result in decreased inhibition of ERK proteins by DUSP1. Phophoinositide-3-phophate kinase may be activated by RAS-GTP. This results in PDK1 activation which phosphorylates AKT. Phosphorylated AKT is channeled into a plethora of downstream biological responses including angiogenesis, cell survival, and cell proliferation. AKT activates IKK. IKK phosphorylates IkB-α, an inhibitor of NF-kB, thus allowing its proteasomal degradation. This results in the activation of NF-kB and its targets. Abbreviations: AKT/PkB, serine/threonine kinase thymoma viral protooncogene; CKS1, CDC28 protein kinase 1b; DUSP1, dual-specificity phosphatase 1; ERK, extracellular signal-regulated kinase; FOXM1, Forkhead box M1B; IKK, Inhibitor of kB kinase; IkB-α, NF-kB inhibitor; MOS, Moloney murine sarcoma viral oncogene homolog; NF-kB, nuclear factor kB; PI3K, phosphatidylinositol-3-kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1; SKP2, S-phase kinase-associated protein 2; uDUSP1, ubiquitinated DUSP1. Pointed and blunt arrows indicate activation and inhibition, respectively.

deletion of p38α kinase enhanced mouse hepatocyte proliferation and chemically induced liver tumorigenesis by antagonizing the JNK/c-JUN pathway (33). Receptor upregulation and overexpression of RAS family genes also stimulate the survival PI3K/AKT pathway (Fig. 3), acting through phosphatidylinositol-3-kinase (PI3K) and generation of phosphoinositol 3,4,5-triphosphate, which activates the serine/threonine kinase thymoma viral protooncogene (AKT/PkB) (34). AKT/PkB inhibits glycogen synthetase kinase-3β (GSK3-β), a constituent, with APC and AXIN, of the β-catenin degradation complex that phosphorylates β-catenin allowing its ubiquitination and degradation. When not degradated, β-catenin forms a cytoplasmic pool of free signaling molecules. Here, stable β-catenin interacts with lymphoid enhancer factor/T cell factor (Lef/Tcf) and is translocated into the nucleus, where it transactivates growth regulatory genes. AKT also causes the release of the proapoptotic molecule BAD from mitochondria and inhibits apoptosis. Finally, AKT blocks, through the mammalian target of rapamycin (mTORC1), the production of the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) and transactivates the 40s ribosomal protein S6kinase (p70s6k), which regulates the transcription of several growth-related genes. The latter mechanism is not active in all HCCs, and stimulation by IGF-1 of *in vitro* proliferation of H4II rat hepatoma cells may also depend on AKT activation independently of the mTORC1 signaling (35). *Akt* upregulation, associated with inactive *Gsk3*-β, occurs in preneoplastic and neoplastic rat liver lesions (Frau *et al*., unpublished results) as well as in HCCs of c-Myc/Tgf-α double transgenic mice (36). Interestingly, recent evidence indicates that upregulation of the PIK3/AKT signaling in human and diethylnitrosamine-induced mouse HCCs is associated with the downregulation of metallothionein expression, suggesting a role for the PIK3/AKT signaling in the regulation of metallothioneins and production of reactive oxygen species (37).

In a recent study we found that moderate activation of Ras, Raf-1, and Mek proteins is paralleled both in susceptible F344 and resistant BN rats by a strong induction of Raf-1, and Mek inhibitors, disabled homolog 2 (Dab2) and Raf kinase inhibitory protein (Rkip), respectively (38). Levels of dual-specificity phosphatase 1 (Dusp1), a specific ERK inhibitor, increases in BN rat lesions, allowing modest ERK activation, whereas a progressive Dusp1 decline occurs in the corresponding lesions of F344 rats and is accompanied by elevated ERK activation (Fig. 3). Furthermore, a gradual increase of Ras association domain family 1A/novel Ras effector 1A/mammalian sterile twenty kinase 1 (RassF1A/Nore1A/Mst1)-driven apoptosis (Fig. 4) was detected in both rat strains. The highest levels being detected in BN rat HCC, whereas loss of Dab2-interacting protein (Dab2IP), a protein implicated in apoptosis signal-regulating kinase 1 (Ask1)-dependent cell death, occurred only in F344 rat HCC. This was associated with significantly higher rates of apoptosis in BN than F344 HCC. Taken together, our results indicate a control of the Ras/Erk pathway and the pro-apoptotic Rassf1A/Nore1A and Dab2IP/Ask1 pathways (Fig. 4) by HCC susceptibility genes. Dusp1 possesses a prominent role in the acquisition of an HCC resistant phenotype by BN rats, whereas late activation of RassF1A/Nore1A and Dab2IP/Ask1 axes is implicated in the highest apoptosis characteristic of BN HCC.

We also evaluated the effects of the functional interactions of ERK proteins with DUSP1 and S-phase kinase-associated protein 2 (SKP2)/CDC28 protein kinase

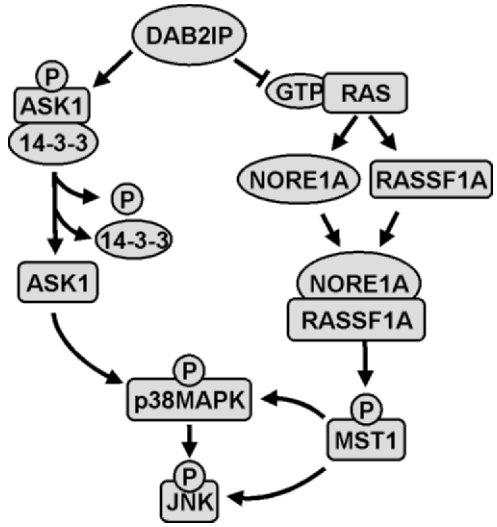

Figure 4. Schematic representation of RASSF1A/NORE1A and DAB2IP/ASK1 pathways leading to cell apoptosis. DAB2IP/ASK1 signaling leads to a fall in the inactive 14-3-3/ASK1 complex. This results in higher availability of free ASK for ASK1-dependent apoptosis. The activation of RaSSF1A/NORE1/MST1 and DAB2IP/ASK1 pathways leads to elevated phosphorylation by pMST1 and/or ASK1 of the proapoptotic proteins p38MAPK and JNK. Abbreviations: ASK1, apoptosis signal-regulating kinase 1; DAB2IP, disabled homolog 2-interacting protein; MST1, mammalian sterile twenty kinase 1; NORE1A, novel Ras effector 1A; RASSF1A, Ras association domain family 1A. Pointed and blunt arrows indicate activation and inhibition, respectively.

1b (CKS1) ubiquitin ligase complex in human HCC (39). Levels of DUSP1, as assessed by real-time reverse transcription PCR and Western blot analysis, were significantly higher in HCCB when compared with both normal and non-tumorous surrounding livers, whereas DUSP1 protein expression sharply declined in all HCCP. In the latter HCC subtype, DUSP1 inactivation was due to either ERK/SKP2/CKS1-dependent ubiquitination (Fig. 3) or promoter hypermethylation associated with loss of heterozygosity at the DUSP1 locus. Noticeably, expression levels of DUSP1 inversely correlated with those of activated ERK as well as with proliferation index and microvessel density, and directly with apoptosis and survival rate. Functional studies revealed that DUSP1 reactivation led to suppression of ERK, CKS1 and SKP2 activity, inhibition of proliferation and induction of apoptosis in human HCC cell lines. Taken together, these observations indicate that ERK achieves unrestrained activity during HCC progression by triggering ubiquitin-mediated proteolysis of its specific inhibitor DUSP1. Therefore, DUSP1 may represent a valuable prognostic marker and ERK, CKS1 or SKP2 are potential therapeutic targets for human HCC.

Recent studies (40) have shown that the activation of Forkhead box M1B (*Foxm1*) and its targets (*AurkA, Cdc2, Cyclin B1, Nek2*) occurs earlier and is most pronounced in liver lesions from F344 than BN rats, leading to highest Cdc2-cyclin B1 complexes (implying highest G2-M transition) in F344 rats. In humans, FOXM1 protein level is progressively induced from surrounding nontumorous liver to HCC, reaching highest levels in tumors with poorer prognosis, and its expression levels directly correlate with proliferation index, genomic instability rate, microvessel density, and inversely correlate with apoptosis (40).

FOXM1 transcription factor is a major downstream effector of ERK (Fig. 3), which triggers the activation of SKP2/CKS1 ubiquitin ligase. SKP2/CKS1 ligase targets $P21^{WAF1}$, $P27^{KIP1}$, and $p57^{KIP2}$ proteins for degradation during the G1-M transition (41). Furthermore, FOXM1 induces the transition of the genes promoting cell cycle progression (*AURKA, CDC2, CYCLIN B1, NEK2,* and *CDC25B*), suppressors of cell cycle inhibitors (*SKP2, CKS1*), and apoptosis inhibitors (*SURVIVIN*) (41–43). *Foxm1* depletion results in block of proliferation and resistance of mouse liver to hepatocarcinogenesis (42–44). The observation that FOXM1 induced SKP2 and CKS1 expression, involved in DUSP1 degradation (38, 39), underlines the implication of FOXM1 in the active proliferation of HCC cells, through a positive feedback loop reinforcing ERK expression, by its ability to inhibit DUSP1 (Fig. 3).

Cell Cycle Deregulation

c-*Myc* overexpression in c-Myc and c-Myc/TGF-α transgenic mice, is associated with deregulation of pRb-E2F pathway (2). Interaction of c-Myc with various cell cycle components occurs in *in vitro* growing HCC cells (45), and c-*Myc* amplification has been found in dysplastic nodules and, more frequently, in carcinomas of rat liver (9, 46). No differences in c-*Myc* expression occur between normal livers of F344 and BN rats (46). However, c-*Myc* expression is much higher in preneoplastic and neoplastic lesions of F344 rats than in the lesions of BN rats. Since c-*Myc* regulates the pRb-E2F pathway, we evaluated cell cycle gene expression in neoplastic nodules and HCCs, induced by initiation/selection protocols, 40 and 70 weeks after diethylnitrosamine treatment, in susceptible F344 rats, and resistant Wistar and BN rats (46). The enzymes controlling cell cycle include cyclin-dependent kinases (CDKs), which are activated by cyclins (47). Complexes of CDK4 and CDK6 with D type cyclins are required for G1 phase progression. Further progression through G1 requires cyclin E, and passage through S phase requires cyclin A, each in complex with CDK2. CDKs activity may be curtailed by various inhibitors including $p16^{INK4A}$, $p27^{KIP1}$, and $p21^{WAF1}$. G1 cyclins (D and E types), in complex with CDKs, phosphorylate retinoblastoma protein (pRb). Hypophosphorylated pRb forms complexes with E2F family transcriptor factors, thus halting cell cycle progression. After pRb phosphorylation, E2F-DP1 complex activates DNA synthesis genes. Comparative analysis of cell cycle regulation in neoplastic nodules and HCC of F344 and BN rats (46) showed overexpression of *c-Myc*, *cyclin D1*, *E*, and *A*, and *E2f1* genes associated with pRb hyperphosphor-

ylation, in the lesions of susceptible F344 rats, and very low or no changes in corresponding lesions from resistant BN rats, compared to normal liver. This indicates a block to G1-S transition in the lesions of resistant strains, which could explain their incapacity to progress. These studies also showed overexpression of the cell cycle inhibitor $p16^{INK4A}$ in fast growing neoplastic tissue of both strains. It was thus hypothesized the existence of interstrain differences in cell cycle protective mechanisms against $p16^{INK4A}$, operated by cell division-cycle 37 (Cdc37), heat shock protein 90 (Hsp90), and required for chromosomal-region-maintenance 1 (Crm1) (48, 49) proteins. Cdc37 forms a chaperone complex with Hsp90, required for the stability and/or activity of diverse protein kinases. E2f4, an essential downstream mediator of $p16^{INK4A}$ acting as a transcription repressor, is largely nuclear in G0 cells, where it is bound to the pocket proteins p107 and p130. It is transported to the cytoplasm by Crm1, as cells emerge into G1 and S (49). In agreement with the above hypothesis, Hsp90, Cdc37, and Crm1 upregulation, decreased presence of the inhibitory $p16^{INK4A}$-Cdk4 complex, and increased levels of Cdc37-Cdk4 and E2f4-Crm1 complexes were found in preneoplastic and neoplastic rat liver (50). These findings strongly suggest the existence of a different, genetically directed modulation of signal transduction pathways, responsible for cell cycle activation, in preneoplastic and neoplastic cells which harbour different susceptibilities to tumor progression, and underline the role of Hsp90/Cdc37 and E2f4/Crm1 systems in the acquisition of a phenotype susceptible or resistant to liver cancer.

Notably, marked upregulation of $P16^{INK4A}$, associated with moderate overexpression of HSP90, CDC37, E2F4 and CRM1 occurs in human HCCB subtype. In contrast, strong induction of HSP90, CDC37 and E2F4 is paralleled by $P16^{INK4A}$ downregulation and high levels of HSP90-CDK4 and CDC37-CDK4 complexes in HCCP (50). *CDC37* gene downregulation by siRNA inhibits *in vitro* growth of HepG2 cells. These results suggest that the protection by CDC37 and CRM1 against growth restraint by $P16^{INK4A}$ may influence the prognosis of human HCC.

Genetic Susceptibility and Epigenetic Regulation of Hepatocarcinogenesis

Labile methyl groups play a pivotal role in liver carcinogenesis. Depletion of methyl groups in rats fed a lipotrope-deficient diet causes cancer or enhances liver tumor development induced by various carcinogens (51). In preneoplastic and neoplastic liver of rats that are fed this diet there is a decrease in S-adenosyl-L-methionine (SAM) and SAM:S-adenosylhomocysteine (SAH) ratio. This is associated with global DNA hypomethylation and overexpression of c-*Jun*, c-*Myc*, and c-H-*Ras*. Similar changes have been found during chemically induced hepatocarcinogenesis, in rats fed diets with adequate content of labile methyl donors (52, 53), as well as in human cirrhosis and HCC (54). A body of evidence indicates that the decrease in SAM content in preneoplastic and neoplastic liver is associated with changes in methionine adenosyltransferase (MAT) isozyme pattern. In mammals, liver-specific *MAT1A* gene encodes MATI/III isozymes, whereas *MAT2A* encodes MATII isozyme, which is widely expressed (54). Fall in *MAT1A* expression and MATI/III activity with concomitant upregulation of *MAT2A* occurs in rodent HCC, human liver cirrhosis and HCC, and human HCC cell lines. Different observations indicate that MAT1A gene expression in human, rat and mouse is correlated with DNA methylation and histone acetylation status. In human HCC, downregulation of *MAT1A* is associated with methylation of CCGG sequences of its promoter and histone H4 deacetylation (55), whereas *MAT2A* upregulation has been attributed to the hypomethylation of the promoter CCGG sequences and H4 histone acetylation (56). Rat *Mat1A* promoter is hypermethylated in embryonic day 19 fetal hepatocytes (57). At this time, *Mat1A* is not expressed, while its expression is detectable immediately before birth. In mouse fetal liver, *Mat1A* gene expression is detectable at embryonic day 14 and increases subsequently (58). The methylation status of 10 CpG sites in the *Mat1A* promoter proximal region has been found to be appreciably correlated with the gene expression in mouse developing liver and in adult hepatic stellate cells and hepatocytes. Furthermore, extremely low *Mat1A* gene expression of mouse hepatoma-derived Hepa-1 cells is enhanced by treatment with the demethylating agent 5-aza-2-deoxycytidine and the deacetylase inhibitor trichostatin A. Conversely, *in vitro* methylation of the *Mat1A* promoter region suppresses the *Mat1A* promoter activity in reporter assay (58). Notably, it was observed that the transcriptional factor CCAAT/enhancer binding protein-β (C/EBP-β) plays an important role in epigenetic regulation of the mature hepatic gene *Mat1A* (58). Indeed, EBP-β specifically binds to a putative binding site of C/EBP-β in the *Mat1A* promoter. C/EBP-β binding controls *Mat1A* promoter activity and is regulated by DNA methylation and histone deacetylation.

MATII isoform is inhibited by its reaction product and its upregulation does not compensate for the fall in MATI/III activity (54). This may lead to a decrease in SAM level, which may be further reduced by increased SAM consumption for polyamine synthesis (59). Indeed, upregulation of genes involved in polyamine synthesis, methionine salvage and downregulation of polyamine negative regulator OAZ1, occurs in HCC and is highest in the aggressive tumors developing in c-Myc/TGF-α HCCs and HCCP (59). Moreover, MATI/III may be inactivated by oxidation of a SH residue of its catalytic portion mediated by $NO^{\bullet}$ overproduction (54). Decrease in SAM levels may reduce its inhibitory effect on cytokine-induced *iNos* gene expression (59), and $NO^{\bullet}$ excess may result in *Ikk* activation, with consequent decrease in IkB-α and rise in NF-kB (27).

A fall in the SAM liver content may enhance tumori-

genesis through multiple mechanisms, including spontaneous oxidative stress, supported by a fall in GSH synthesis, and genomic instability induced by DNA hypomethylation (10, 53, 59, 60). Recent observations (61) indicate the existence of an epigenetic modulation of the process of carcinogenesis by ROS. ROS induction in human HCC tissues is associated with up-regulation of Snail, a transcription factor that down-regulates E-cadherin expression. Snail induces DNA methylation of the E-cadherin promoter by recruiting histone deacetylase 1 and DNA methyltransferase 1. These findings could be relevant to understand the activity of ROS in silencing various tumor suppressor genes during tumorigenesis.

Differential expression of *MAT1A* and *MAT2A* genes influences DNA methylation and growth of human HCC (54, 58). Notably, human studies revealed a direct correlation between genomic instability and cell growth in human HCC, and an inverse correlation between genomic instability and survival rate (58). Moreover, DNA methylation is inversely correlated with genomic instability (58), and we recently found an inverse correlation between *MAT1A:MAT2A* ratio and HCC proliferation as well as a direct correlation with survival of HCC patients (Frau *et al*., unpublished results). These findings assign a putative prognostic role to the *MAT1A:MAT2A* ratio during hepatocarcinogenesis.

The effect of susceptibility/resistance genes on DNA methylation in HCC has not been explored as yet. Recent results in our laboratory (Frau *et al*., unpublished data) have shown that HCCs of BN rats, in contrast to F344 rat lesions, do not exhibit global DNA hypomethylation and decrease in *Mat1A:Mat2A* ratio, or reduced levels of SAM. This is associated with the absence of significant changes in the methylation of CpG islands of *Mat1A* and *Mat2A* promoters in HCC. In contrast, CpG islands of *Mat1A* promoter are prevalently methylated and those of *Mat2A* promoter are hypomethylated in susceptible F344 rats. This may explain the decrease in *Mat1:Mat2A* expression ratio in F344 HCCs, suggesting the interference of HCC susceptibility/resistance genes with the epigenetic control of *Mat1A* and *Mat2A* gene expression. Further work is necessary to clarify the mechanisms underlying the control of susceptibility genes on promoter methylation for *Mat1A* and *Mat2A* genes.

Concluding Remarks

HCC is a rapidly fatal disease, with a life expectancy of about 6 months from the time of the diagnosis. Therapeutic strategies employed to date have significantly improved the prognosis for patients with unresectable HCC (1–3, 10). This emphasizes the need for investigating the molecular mechanisms responsible for HCC development in order to identify new targets for early diagnosis, chemoprevention, and treatment. However, the complexity of molecular changes in HCC predicts lack of complete therapeutic efficacy for strategies relying on interference with a single signaling pathway. To overcome this difficulty and to prevent development of resistance to single agents therapies, networked biologic therapies have been proposed in which a combination of non-cytotoxic interventions must be performed (32, 62). These interventions may be directed so as to interfere with different cell survival pathways, enhance apoptosis, block angiogenesis and extrahepatic fibrosis, induce the lysis of tumor cells, stimulate antitumor immunity, decrease HBV and HCV replication, etc. The combination of gene therapy with conventional therapeutic approaches using cytotoxic drugs may also improve the treatment and reduce the doses of toxic compounds.

Aberrant activation of several signaling pathways, including the EGFR, ERK, Pi3K/AKT, c-met/HGF, Wnt, Hedgehog and apoptosis signaling, occurs in human HCCs (61). Various studies have attempted to block some of these signaling cascades or to interfere with VEGF and its receptor (32). There is little information on the efficacy of combination therapies so far. However, the advent of targeted therapies for HCC has underscored the importance of identifying novel molecular targets for tumor treatment, including the evaluation of the contribution of signaling pathways to tumor cell proliferation in different HCC subtypes, selected according to their clinical and pathologic features.

Research on polygenic predisposition to HCC has led to the identification of genetic factors determining the individual predisposition to hepatocarcinogenesis, which is in turn determined by the capacity of preneoplastic and neoplastic lesions to grow and progress to HCC (10). Thus, the identification of the signaling cascades, affected by susceptibility/resistance genes, might give insights on key genes responsible for the presence of a phenotype favourable to HCC development and progression. Our recent studies indicate that this represents a useful approach to discover prognostic markers and therapeutic targets for HCC. Putative prognostic markers, among genes whose activity is controlled by susceptibility/resistance genes, include global DNA methylation (58) and *DUSP1* (39), exhibiting positive correlation with survival time in HCC patients. There is also evidence for a positive correlation of iNOS (28), FOXM1 (40), and SKP2 (Frau, unpublished results) expression with proliferation index of HCC, and a negative correlation with patients' survival length. Furthermore, the knock-down of genes influenced by genetic susceptibility to liver cancer, including c-*MYC, CDC7, SKP2*, or *CSK1*, by specific siRNAs, or *ERK1*/*2*, by siRNA or the inhibitor compound UO126, strongly inhibits the proliferation and induces apoptosis in human HCC cell lines (39).

Another important aspect of recent research on signaling pathways, involved in hepatocarcinogenesis, is the discovery that most of the alterations responsible for the acquisition of a resistant or susceptible phenotype in rats, also have a similar contribution to the prognosis, of human HCC. Studies on families at risk and segregation studies on human population (11, 63, 64) support a genetic model of

predisposition to hepatocarcinogenesis (10) with a major locus and various low-penetrance genes, at play in different subsets of population, similar to the genetic model controlling hepatocarcinogenesis in rodents. Further studies are needed to clarify the influence of susceptibility genes on signaling pathways supporting tumor growth and progression in humans. However, since early blockade of signaling pathways may result in more efficient prevention, rodent models of hepatocarcinogenesis may be useful in identifying progression markers and therapeutic targets in early stages of the process, and in a large number of HCC subtypes. The study of experimental models recapitulating early preneoplastic alterations of human liver carcinogenesis may lead to the discovery of biomarkers of the risk of cirrhosis evolution to full malignancy, as well as of new regulatory genes and signal transduction pathways involved in hepatocarcinogenesis.

1. Bruix J, Boix L, Sala M, Llovet, JM. Focus on hepatocellular carcinoma. Cancer Cell 5:215–219, 2004.
2. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 31:339–346, 2002.
3. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 6:674–678, 2006.
4. Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, Alter HJ. A comparison of the molecular clock of hepatitis C virus in United States and Japan predicts that hepatocellular carcinoma incidence in United States will increase over the next two decades. Proc Natl Acad Sci U S A 99:15584–15589, 2002.
5. Sell S. Cellular origin of hepatocellular carcinoma. Cell Dev Biol 13: 419–424, 2002.
6. Su Q, Bannasch P. Relevance of hepatic preneoplasia for human hepatocarcinogenesis. Toxicol Pathol 31:126–133, 2003.
7. Farber E, Sarma DSR. Hepatocarcinogenesis: a dynamic cellular perspective. Lab Invest 56:4–22, 1987.
8. Kern MA, Breuhahn K, Schirmacher P. Molecular pathogenesis of human hepatocellular carcinoma. In: Vance Woude GF, Kelin G, Eds. Advances in Cancer Research. New York: Academic Press, Vol 86: pp70–111, 2002.
9. Feo F, Pascale RM, Simile MM, De Miglio MR, Muroni MR, Calvisi DF. Genetic alterations in liver carcinogenesis. Crit Rev Oncog 11:19–62, 2000.
10. Feo F, De Miglio MR, Simile MM, Muroni MR, Calvisi DF, Frau M, Pascale RM. Hepatocellular carcinoma as a complex polygenic disease. Interpretative analysis of recent developments on genetic predisposition. BBA Cancer Rev 1765:126–147, 2006.
11. Cai RL, Meng W, Lu HY, Lin WY, Jiang F, Shen FM. Segregation analysis of hepatocellular carcinoma in a moderately high-incidence area of East China. World J Gastroenterol 9:2428–2432, 2003.
12. Dragani TA, Manenti G, Della Porta G. Genetic susceptibility to murine hepatocarcinogenesis is associated with high growth rate of NDEA-initiated hepatocytes. J Cancer Res Clin Oncol 113:223–229, 1987.
13. Dragani TA, Manenti G, Gariboldi M, De Gregorio L, Pierotti MA. Genetics of liver tumor susceptibility in mice. Toxicol Lett 82–83:613–619, 1995.
14. Bilger A, Bennet LM, Carabeo RA, Chiaverott TA, Dvorak C, Liss KM, Schadewald SA, Pitot HC, Drinkwater NR. A potent modifier of liver cancer risk on distal mouse chromosome 1: linkage analysis and characterization of congenic lines. Genetics 167:859–866, 2004.
15. Poole TM, Drinkwater NR. Two genes abrogate the inhibition of murine hepatocarcinogenesis by ovarian hormones. Proc Natl Acad Sci U S A 93:5848–5853, 1996.
16. Lee GH, Drinkwater NR. The Hcr (hepatocarcinogen resistance) loci of DBA/2J mice partially suppress phenotypic expression of the Hcs (hepatocarcinogen sensitivity) loci of C3H/HeJ mice. Carcinogenesis 161:1993–1996, 1995.
17. Manenti G, Galvan A, Favella FS, Pascale RM, Spada E, Dilani S, Neira AG, Feo F, Dragani TA. Genetic control of resistance to hepatocarcinogenesis by the mouse Hpcr3 locus. Hepatology 48:617–623, 2008.
18. Melhem MF, Kunz HW, Gill TJ III. A major histocompatibility complex-linked locus in the rat critically influences resistance to diethylnitrosamine carcinogenesis. Proc Natl Acad Sci U S A 90:1967–1971, 1993.
19. De Miglio MR, Canzian F, Pascale RM, Simile MM, Muroni MR, Calvisi DF, Romeo G, Feo F. Identification of genetic loci controlling hepatocarcinogenesis on rat chromosomes 7 and 10. Cancer Res 59: 4651–4657, 1999.
20. De Miglio MR, Pascale RM, Simile MM, Muroni MR, Calvisi DF, Virdis P, Bosinco GM, Frau M, Seddaiu MA, Ladu S, Feo F. Chromosome mapping of multiple loci affecting the genetic predisposition to rat liver carcinogenesis. Cancer Res 62:4459–4463, 2002.
21. De Miglio MR, Pascale RM, Simile MM, Muroni MR, Virdis P, Kwong KM-T, Wong LKL, Bosinco GM, Pulina FR, Calvisi DF, Frau M, Wood GA, Archer MC, Feo F. Polygenic control of hepatocarcinogenesis in Copenhagen x F344 rats. Int J Cancer 111:9–16, 2004.
22. Zeng ZZ, Higashi S, Kitayama W, Denda A, Yan Y, Matsuo K, Konishi Y, Hiai H, Higashi K. Genetic resistance to chemical carcinogen-induced preneoplastic hepatic lesions in DRH strain rats. Cancer Res 60:2876–2881, 2000.
23. Liu H, Higashi K, Hiai H. Role of resistant Drh1 locus in chemical carcinogen-induced hepatocarcinogenesis in rats: analysis with a speed congenic strain. Cancer Sci 9:164–169, 2005.
24. De Miglio MR, Virdis P, Calvisi DF, Frau M, Muroni MR, Simile MM, Daino L, Careddu G, Sanna-Passino E, Pascale RM, Feo F. Mapping a sex-hormone sensitive gene determining female resistance to liver carcinogenesis in a congenic F344.BN-*Hcs4* rat. Cancer Res 66:10384–10390, 2006.
25. Kawanishi S, Hiraku Y, Pinlaor S, Ma N. Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to inflammation-related carcinogenesis. Biol Chem 387:365–372, 2006.
26. Shibutani S, Takeshita M, Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature 349:431–434, 1991.
27. Simile MM, Pagnan G, Pastorino F, Brignole C, De Miglio MR, Muroni MR, Asara G, Frau M, Seddaiu MA, Calvisi DF, Feo F, Ponzoni M, Pascale RM. Chemopreventive N-(4-hydroxyphenyl)retinamide (fenretinide) targets deregulated NF-{kappa}B and Mat1A genes in the early stages of rat liver carcinogenesis. Carcinogenesis 26: 417–422, 2005.
28. Calvisi DF, Pinna F, Ladu S, Pellegrino R, Muroni MR, Simile MM, De Miglio MR, Frau M, Tomasi ML, Seddaiu MA, Daino L, Virdis P, Feo F, Pascale RM. Aberrant iNOS signaling is under genetic control in rodent liver cancer and potentially prognostic for human disease. Carcinogenesis 29:1639–1647, 2008.
29. Arsura M, Cavin LG. Nuclear factor-kB and liver carcinogenesis. Cancer Lett 229:157–169, 2005.
30. Hussain PS, Hofset LJ, Harris CC. Radical causes of cancer. Nat Rev Cancer 3:276–285, 2003.
31. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 410:37–40, 2001.
32. Calvisi DF, Pascale RM, Feo F. Dissection of signal transduction pathways as a tool for the development of targeted therapies of hepatocellular carcinoma. Rev Recent Clin Trials 2:217–236, 2007.

33. Hui L, Bakiri L, Mairhorfer A, Schweifer N, Haslinger C, Kenner L, Komnenovic V, Scheuch H, Beug H, Wagner EF. p38alpha suppresses normal and cancer cell proliferation by antagonizing the JNK-c-Jun pathway. Nat Genet 39:741–749, 2007.
34. Woodgett JR. Recent advances in the protein kinase B signaling pathway. Curr Opin Cell Biol 17:150–157, 2005.
35. Alexia C, Fourmatgeat P, Delautier D, Groyer A. Insulin-like growth factor-I stimulates H4II rat hepatoma cell proliferation: dominant role of PI-3′K/Akt signaling. Exp Cell Res 312:1142–1152, 2006.
36. Factor V, Oliver AL, Panta GR, Thorgeirsson SS, Sonenshein GE, Arsura M. Roles of Akt/PKB and IKK complex in constitutive induction of NF-kB in hepatocellular carcinomas of transforming growth factor alpha/c-myc transgenic mice. Hepatology 34:32–41, 2001.
37. Datta J, Majumder S, Kutay H, Motiwala T, Frankel W, Costa R, Cha HC, MacDougald OA, Jacob ST, Ghoshal K. Metallothionein expression is suppressed in primary human hepatocellular carcinomas and is mediated through inactivation of CCAAT/enhancer binding protein alpha by phosphatidylinositol 3-kinase signaling cascade. Cancer Res 67:2736–2746, 2007.
38. Calvisi DF, Pinna F, Ladu S, Pellegrino R, Sanna V, Sini M, Daino L, Simile MM, De Miglio MR, Frau M, Tomasi ML, Seddaiu MA, Muroni MR, Feo F, Pascale RM. Ras-driven proliferation and apoptosis signaling during rat liver carcinogenesis is under genetic control. Int J Cancer 123:2057–2064, 2008.
39. Calvisi DF, Pinna F, Meloni F, Ladu S, Pellegrino R, Sini M, Daino L, Simile MM, De Miglio MR, Virdis P, Frau M, Tomasi ML, Seddaiu MA, Muroni MR, Feo F, Pascale RM. Dual-Specificity phosphatase 1 ubiquitination in extracellular signal-regulated-kinase-mediated control of growth in human hepatocellular carcinoma. Cancer Res 68:4192–4200, 2008.
40. Calvisi DF, Pinna F, Ladu S, Pellegrino R, Simile MM, Frau M, De Miglio MR, Tomasi ML, Sanna V, Muroni MR, Feo F, Pascale RM. Forkhead box M1B is a determinant of rat susceptibility to hepatocarcinogenesis and sustains ERK activity in human HCC. Gut 2008, in press.
41. Costa RH, Kalinichenko VV, Major ML, Raychaudhuri P. New and unexpected: forkhead meets ARF. Curr Opin Genet Dev 15:42–48, 2005.
42. Wierstra I, Alves J. FOXM1, a typical proliferation associated transcription factor. Biol Chem 388:1257–1274, 2007.
43. Wang X, Kiyokawa H, Dennewitz MB, Costa RH. The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. Proc Natl Acad Sci U S A 99: 16881–16886, 2002.
44. Kalinichenko VV, Major ML, Wang X, Petrovic V, Kuechle J, Yoder HM, Dennewitz MB, Shin B, Datta A, Raychaudhuri P, Costa RH. Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19ARF tumor suppressor. Genes Dev 18:830–850, 2004.
45. Nasi S, Ciarapica R, Jucker R, Rosati J, Souceck L. Making decisions through Myc. FEBS Lett 490:153–162, 2001.
46. Pascale RM, Simile MM, De Miglio MR, Muroni MR, Calvisi DF, Asara G, Casabona D, Frau M, Seddaiu MA, Feo F. Differences in cell cycle deregulation in neoplastic liver lesions of rats with and without genetic predisposition to hepatic carcinogenesis. Hepatology 15:1341–1350, 2002.
47. Adams PD. Regulation of retinoblastoma tumor suppressor protein by cyclin/cdks. Biochim Biophys Acta 1471:123–133, 2001.
48. Stepanova L, Finegold M, DeMayo F, Schmidt EV, Harper JW. The oncoprotein kinase chaperone CDC37 functions as an oncogene in mice and collaborates with both c-myc and cyclin D1 in transformation of multiple tissues. Mol Cell Biol 20:4462–4473, 2000.
49. Gaubatz S, Lees JA, Lindeman GJ, Livingston DM. E2F4 is exported from the nucleus in a CRM1-dependent manner. Mol Cell Biol 21: 1384–1392, 2001.
50. Pascale RM, Simile MM, Calvisi DF, Frau M, Muroni MR, Seddaiu MA, Daino L, Muntoni MD, De Miglio MR, Thorgeirsson SS, Feo F. Role of HSP90, CDC37, and CRM1 as modulators of P16(INK4A) activity in rat liver carcinogenesis and human liver cancer. Hepatology 42:1310–1319, 2005.
51. Wainfan E, Poirier LA. Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. Cancer Res 52:2071s–2077s, 1992.
52. Garcea R, Daino L, Pascale RM, Simile MM, Puddu M, Ruggin ME, Seddaiu MA, Satta G, Sequenza MJ, Feo F. Protooncogene methylation and expression in regenerating liver and preneoplastic liver nodules induced in the rat by diethylnitrosamine: effect of variations of S-adenosylmethionine: S-adenosylhomocysteine ratio. Carcinogenesis 10:1183–1192, 1989.
53. Rao PM, Antony A, Rajalakshmi S, Sarma DS. Studies on hypomethylation of liver DNA during early stages of chemical carcinogenesis in rat liver. Carcinogenesis 10:933–937, 1989.
54. Mato JM, Corrales FJ, Lu SC, Avila MA. S-Adenosylmethionine: a control switch that regulates liver function. FASEB J 16:15–26, 2002.
55. Torres L, Avila MA, Carretero MV, Latasa MU, Caballería J, López-Rodas G, Boukaba A, Lu SC, Franco L, Mato JM. Liver-specific methionine adenosyltransferase MAT1A gene expression is associated with a specific pattern of promoter methylation and histone acetylation: implications for MAT1A silencing during transformation. FASEB J 14: 95–102, 2000.
56. Yang H, Huang ZZ, Zeng Z, Chen C, Selby RR, Lu SC. Role of promoter methylation in increased methionine adenosyltransferase 2A expression in human liver cancer. Am J Physiol Gastrointest Liver Physiol 280:G184–190, 2001.
57. Torres L, Lopez-Rodas G, Latasa MU, Carretero MV, Boukaba A, Rodriguez JL, Luis Franco L, Mato JM, Avila MA. DNA methylation and histone acetylation of rat methionine adenosyltransferase 1A and 2A genes is tissue-specific. Int J Biochem Cell Biol 32:397–404, 2000.
58. Ikeda R, Nishida T, Watanabe F, Shimizu-Saito K, Asahina K, Horikawa S, Teraoka H. Involvement of CCAAT/enhancer binding protein-beta (C/EBPbeta) in epigenetic regulation of mouse methionine adenosyltransferase 1A gene expression. Int J Biochem Cell Biol 40: 1956–1969, 2008.
59. Calvisi DF, Simile MM, Ladu S, Pellegrino R, De Murtas V, Pinna F, Tomasi ML, Frau M, Virdis P, De Miglio MR, Muroni MR, Pascale RM, Feo F. Altered methionine metabolism and global DNA methylation in liver cancer: relationship with genomic instability and prognosis. Int J Cancer 121:2410–2420, 2007.
60. Majano PL, Garcia-Monzon C, García-Trevijano ER, Corrales FJ, Camara J, Ortiz P, Mato JM, Avila MA, Moreno-Otero R. S-adenosylmethionine modulates inducible nitric oxide synthase gene expression in rat liver and isolated hepatocytes. J Hepatol 35:692–699, 2001.
61. Lim S-O, Gu JM, Kim MS, Kim H-S, Park YN, Park CK, Cho JW, Park YM, Jung G. Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. Gastroenterology 135:2128–2140, 2008.
62. Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. Hepatology 48:1312–1327, 2008.
63. Fernandez E, La Vecchia C, D'Avanzo B, Negri E, Franceschi S. Family history and the risk of liver, gallbladder, and pancreatic cancer. Cancer Epidemiol Biomarkers Prev 3:209–212, 1994.
64. Hemminki K, Li X. Familial risks of cancer as a guide to gene identification and mode of inheritance. Int J Cancer 110:291–294, 2004.