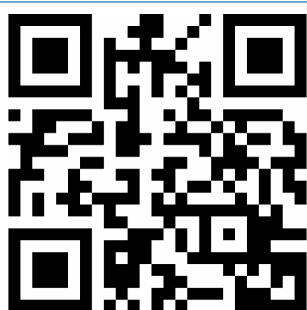


New insights on the role of epigenetic alterations in hepatocellular carcinoma

Maddalena Frau¹
 Claudio F Feo²
 Francesco Feo¹
 Rosa M Pascale¹

¹Department of Clinical and Experimental Medicine, Division of Experimental Pathology and Oncology, ²Department of Clinical and Experimental Medicine, Division of Surgery, University of Sassari, Sassari, Italy

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Abstract: Emerging evidence assigns to epigenetic mechanisms heritable differences in gene function that come into being during cell development or via the effect of environmental factors. Epigenetic deregulation is strongly involved in the development of hepatocellular carcinoma (HCC). It includes changes in methionine metabolism, promoter hypermethylation, or increased proteasomal degradation of oncosuppressors, as well as posttranscriptional deregulation by microRNA or messenger RNA (mRNA) binding proteins. Alterations in the methylation of the promoter of methyl adenosyltransferase *MAT1A* and *MAT2A* genes in HCC result in decreased S-adenosylmethionine levels, global DNA hypomethylation, and deregulation of signal transduction pathways linked to methionine metabolism and methyl adenosyltransferases activity. Changes in S-adenosylmethionine levels may also depend on *MAT1A* mRNA destabilization associated with *MAT2A* mRNA stabilization by specific proteins. Decrease in *MAT1A* expression has also been attributed to miRNA upregulation in HCC. A complex deregulation of miRNAs is also strongly involved in hepatocarcinogenesis, with up-regulation of different miRNAs targeting oncosuppressor genes and down-regulation of miRNAs targeting genes involved in cell-cycle and signal transduction control. Oncosuppressor gene down-regulation in HCC is also induced by promoter hypermethylation or posttranslational deregulation, leading to proteasomal degradation. The role of epigenetic changes in hepatocarcinogenesis has recently suggested new promising therapeutic approaches for HCC on the basis of the administration of methylating agents, inhibition of methyl adenosyltransferases, and restoration of the expression of tumor-suppressor miRNAs.

Keywords: hepatocarcinogenesis, DNA methylation, microRNA, Piwi-interacting RNAs, stem cells, therapeutic targets

Introduction

Hepatocellular carcinoma (HCC) is a frequent and deadly human cancer, with 0.25–1 million new cases per year and a life expectancy of about 6 months from the time of diagnosis.^{1,2} HCC incidence changes with age, sex, ethnic group, and geographic region³ and is rising in Western countries.^{4,5} Major risk factors associated with HCC development are chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alcoholic steatohepatitis, Aflatoxin B1,^{1–3} and some metabolic diseases.⁶ HCC incidence shows differences within human population in response to risk factors,⁷ suggesting a pathogenetic role of genetic predisposition. Studies on genetic predisposition of chemically induced rodent hepatocarcinogenesis and of human families at risk suggest the implication of a polygenic control of HCC incidence.^{8,9} The interaction of genetic and environmental risk factors creates a wide genotypic and phenotypic heterogeneity within human HCC.¹⁰

Correspondence: Francesco Feo
 Department of Clinical and Experimental Medicine, University of Sassari, Division of Experimental Pathology and Oncology, Via P Manzella 4, 07100 Sassari, Italy
 Tel +35 079 228307
 Fax +35 079 228305
 Email feo@uniss.it



A body of evidence indicates that the interaction of DNA with carcinogens and reactive radicals, generated during carcinogen metabolism and/or inflammation in early phases of liver carcinogenesis, induces genomic instability (GI) with consequent genomic alterations and signaling pathway deregulation.^{2,8,11,12} This allows initiated cells to evolve to dysplastic nodules and malignant lesions.^{8,11,12}

This review provides an analysis of the epigenetic mechanisms implicated in signaling pathway deregulation during hepatocarcinogenesis. We explore the contribution of epigenetic changes to identify new putative prognostic markers and opportunities for targeted therapies.

Role of epigenetics

Epigenetics refers to a gene activity stable over long time that may be inherited and reprogrammed, affecting gene function, during cell development or as a consequence of environmental factors, without any change of DNA sequence. Epigenetic mechanisms include DNA methylation, nucleosome remodeling, histone modification (acetylation, methylation of arginine or lysine residues, and ubiquitination), mRNA and protein turnover, and small and microRNA (miRNA) noncoding sequences. These processes contribute to control gene function by affecting DNA structure, DNA-binding protein architecture, DNA access to transcription factors, and RNA and protein degradation.

DNA methylation

One of the best-understood molecular mechanisms of epigenetic gene function control involves methylation of specific cytosine lining upstream of a promoter region. The methylation of cytosine residues of CpG dinucleotides changes the major trench of the DNA chain, thus altering the proteins and transcription factors binding to DNA.

Around 4%–8% of DNA cytosines are methylated, mostly in CpG sequences.¹³ DNA-methyltransferases (DNMTs) catalyze DNA methylation, using S-adenosylmethionine (SAM) as methyl donor. DNMT-1 acts as maintenance methylase during cell replication and, using the parental methylated DNA strand as template, provides the epigenetic information between cell generations.¹⁴ DNMT3a and DNMT3b are responsible for de novo methylation during development.¹⁵ The presence of the methyl group directly interferes with the binding of a protein to its cognate DNA sequence. Many transcription factors fail their binding ability when the CpGs are methylated, leading to transcription repression.¹⁶ Significant correlations between loss of E-cadherin, resulting from promoter methylation, and tumor stage and location have been

observed in a subset of gastric cancer patients, supporting a correlation between promoter hypermethylation and tumor aggressiveness and metastasis.¹⁷

The mechanisms limiting genomic methylation to a minority of CpG islands are poorly understood. A simple mechanism creating an unmethylated DNA domain is the presence into a DNA stretch of proteins causing DNMT exclusion.¹⁸ According to recent observations,¹⁹ RNA arising from the *C/EBPA* (CCAAT/enhancer binding protein α) transcription factor gene locus binds to DNMT-1 and prevents *C/EBPA* gene locus methylation. These interesting findings suggest that the interaction between DNMT-1 and RNA plays a role in selective DNA hypomethylation.

Chromatin and histone modification

An additional mechanism of epigenetic control is the association of histone proteins with DNA. Chromatin formation and the chemical modification of chromatin by covalent changes, as acetylation, arginine and/or lysine methylation, phosphorylation, SUMOylation and ubiquitination, can change DNA accessibility. Hypo-acetylation of lysine residues on core histone H4 is coupled with chromatin compaction and gene repression, while euchromatin displays higher levels of histone acetylation.²⁰ The methylation of arginine or lysine residues does not alter the charge of histones but, depending on which arginine or lysine residues are modified, it can positively or negatively affect gene expression.²¹ Chromatin heavily acetylated, forming the so called “open configuration”, allows transcription factors to interact with gene promoters.²² Chemical marks in the N/C terminal tails of histone proteins are effectors of gene modulations, they influence also gene splicing, and DNA replication, repair, and recombination.

mRNA decay and protein turnover

An important molecular mechanism involved in the modification of gene expression is the mRNA decay process. Several signal transduction pathways contribute to controlling mRNA stability. The lability of mRNAs codifying signal transduction factors, cytokines, and oncoproteins contributes to determine their required levels in accordance with endogenous or exogenous stimuli. Most mRNAs start their decay by shortening their poly(A)-tail. Deadenylated mRNA that loses its 5'-cap (decapping) becomes suitable for digestion by exonucleases.²³

mRNA instability is determined by AREs (elements rich of Au dinucleotides in the untranslated regions [UTRs]). AREs generally promote rapid mRNA degradation

by deadenylation. They are grouped into class I, containing one or more AUUUA sequences in the U-rich region; class II, characterized by AUUUA tandem repeats; and U-rich class III, devoid of AUUUA. mRNAs encoding oncoproteins contain class I and class II sequences. Cytokines and chemokines encoding mRNAs often contain class II ARE sequences. AREs should interact with ARE-interacting proteins (ARE-BP) to regulate mRNA stability. ARE-BPs include AUF1/hnRNP, Hsc70, Hsp70 HuH/Hur, and several other proteins.²³ RNA interaction with specific elements can confer transcript stability. Stable transcripts encode housekeeping proteins. Phosphorylation (ensured by activated protein kinases, such as p38MAPK, ERK, and Wnt/ β -catenin), isomerization, methylation, and relocalization are ways to modulate RNA binding to proteins and, consequently, contribute to coordinating gene expression.

Noncoding RNA

In recent years, much attention focused on noncoding RNA (ncRNA), a family of untranslated transcripts monoallelically expressed, deriving from the classically denominated “junk” DNA. ncRNA may be distinguished into long ncRNA (>200 nucleotides), transcribed by RNA polymerase II, and short ncRNA (20–40 nucleotides). Other species less well-characterized also may exist. Long ncRNA positioned in cis within large chromatin domains is involved in silencing multiple genes.²⁴ Short ncRNAs are represented by miRNAs and Piwi-interacting RNAs.

miRNAs are noncoding minute RNAs implicated in post-transcriptional regulation of gene expression (Figure 1).

In the nucleus, RNA polymerase II transcribes primary miRNAs (pri-miRNAs) constituted by 5'-7-methyl guanylate cap, stem-loop secondary structures, and a 32-poly(A) tail.^{25,26}

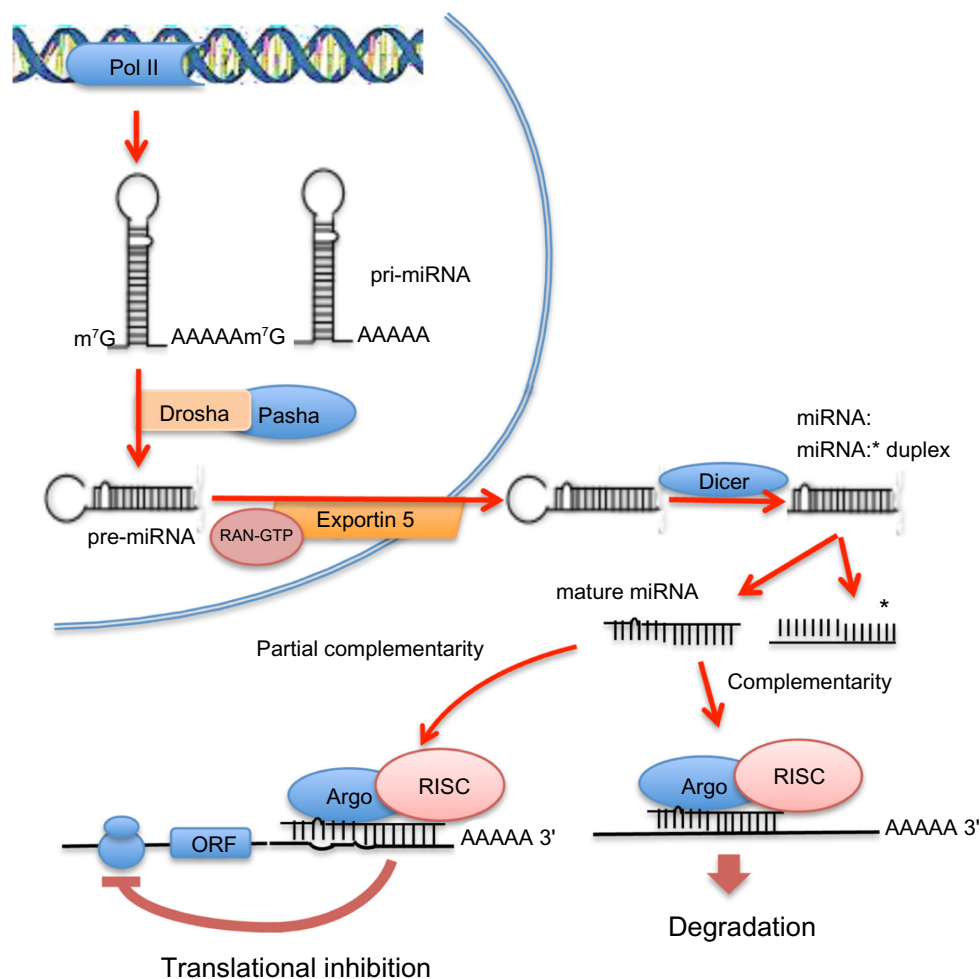


Figure 1 Intranuclear and extranuclear phases of the process of maturation of microRNAs (miRNAs) and mechanisms of inhibition of messenger RNA transcription. In the nucleus, long primary transcripts (primary miRNAs) are cleaved by RNase III Drosha, with its cofactor Pasha, to release the precursor miRNAs, which are exported to the cytoplasm by exportin 5. In the cytoplasm, precursor miRNAs are processed by RNase III Dicer, with the release double-stranded duplexes of 20–23 RNA that contain the mature miRNA and the passenger miRNA strand (asterisked). The mature miRNAs interact with the 3' untranslated region of its targeted mRNAs to form, with Argonaute proteins, the RNA-induced silencing complex (RISC). RISC binds to the 3' untranslated region of its target or targets, inhibiting translation of single or multiple proteins; complete miRNA complementarity induces mRNA degradation, whereas partial complementarity represses translation.

Abbreviations: RAN, RAS-related nuclear protein; GTP, guanosine-5'-triphosphate; ORF, open reading frame.

The endoribonuclease III, Drosha, with its cofactor Pasha, cleaves pri-miRNAs into 50–150 nucleotide precursor miRNAs, which are exported to cytoplasm by Exportin 5. In the cytoplasm, RNase III Dicer cleaves precursor miRNAs into 20–23 nucleotide double-stranded duplexes. Each duplex splits into single-stranded mature miRNA, which associates with Argonaute proteins constituting the RNA-induced silencing complex (RISC). RISC binds to the 3'-UTR of its mRNA target or targets, inhibiting their translation.²⁷ Totally complementary miRNAs induce mRNA degradation, whereas partial complementarity inhibits translation. A body of evidence assigns to miRNAs a key role in the regulation of gene expression. miRNA deregulation is strongly involved in cancer development.²⁸

Piwi-interacting RNAs silence gene functions through base-pairing with mRNA 3'-UTRs. They can either repress translation or determine mRNA degradation by a RNAi (interference)-like mechanism. Piwi-interacting RNAs constitute a novel class of small RNAs that specifically protect mammalian DNA germline from transposon activity.²⁹

The disruption of epigenetic regulatory mechanisms in oncogenesis may lead to inappropriate expression of genes regulating key cellular processes.³⁰ Cancer cell genome is often globally hypomethylated, with a loss of 20%–60% of 5'-methylcytosine leading to activation of previously repressed protooncogenes. Several oncosuppressor genes are frequently hypermethylated in cancer. DNA repair genes may

be subjected to promoter methylation. Cancers with hypermethylated methylguanosintransferase genes are susceptible to *p53* and *K-ras* mutations.³¹ Hypermethylation of *MLH1* (*MutL*, *Escherichia coli*, homolog of, 1), a mismatch repair gene, can cause microsatellite instability in colorectal and endometrial cancers.³² A clear example of epigenetic–genetic cooperation occurs in the colon cancer cell line HCT116, in which one allele of *MLH1* and *CDKN2A* (cyclin-dependent kinase inhibitor 2a) genes is mutated and the other allele is silenced by DNA methylation, with consequent functional loss of DNA mismatch repair and cell cycle regulation.³³

Epigenetic deregulation in hepatocellular carcinoma

Different observations showed global DNA hypomethylation and steatohepatitis, followed by HCC development even without carcinogens administration, in rats fed methyl-deficient diets (MDDs).^{34–36} MDDs induce a fall in SAM, the major methyl donor, and in SAM/S-adenosylhomocysteine (SAH) ratio. Remarkably, persistent decrease in SAM content and SAM/SAH ratio was also found in the liver, dysplastic nodules, and HCC of rats fed an adequate diet and subjected to the administration of different carcinogens in various experimental models.^{37–41} Global DNA hypomethylation and SAM decrease, with no change in SAH, was also found in human HCC and, to a lesser extent, in cirrhotic liver.⁴² These

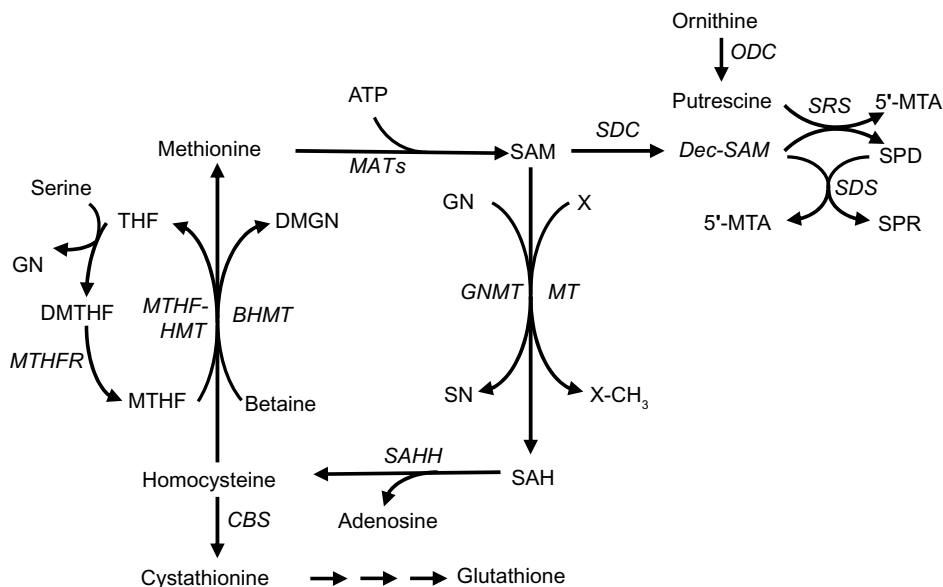


Figure 2 Methionine metabolism.

Abbreviations: ODC, ornithine decarboxylase; ATP, adenosine triphosphate; SRS, spermine synthase; 5'-MTA, 5'-methylthioadenosine; SDC, SAM decarboxylase; SAM, S-adenosylmethionine; Dec-SAM, decarboxylated SAM; SPD, spermidine; MAT, methionine adenosyltransferase; SDS, spermidine synthase; THF, tetrahydrofolate; DMGN, dimethyl GN; GN, glycine; SPR, spermine; DMTHF, dimethyl-THF; MTHF-HMT, 5-methyltetrahydrofolate homocysteine methyltransferase; BHMT, betaine-homocysteine methyltransferase; GNMT, glycine N-methyltransferase; MT, methyltransferase; MTHFR, methyltetrahydrofolate reductase; MTHF, methyl-THF; SN, sarcosine; X-CH₃, methylated compounds; SAHH, SAH hydrolase; SAH, S-adenosylhomocysteine; CBS, cystathionine beta-synthase.

observations associate HCC development with global DNA hypomethylation and decrease in SAM.

Deregulation of methionine metabolism and global DNA hypomethylation in HCC

Liver is the main source of SAM, which is synthesized from methionine and adenosine triphosphate (ATP) in a reaction catalyzed by methionine adenosyltransferases (MATs)^{42,43} (Figure 2). SAM may undergo decarboxylation, followed by polyamine synthesis, or conversion to SAH throughout transmethylation reactions. SAH hydroxylase (SAHH) catalyzes the conversion of SAH to homocysteine and adenosine. Homocysteine is a precursor of GSH (reduced glutathione) via the transsulfuration pathway. Alternatively, betaine-homocysteine methyltransferase (BHMT) catalyzes the synthesis of methionine and dimethylglycine from homocysteine and betaine, whereas 5-methyltetrahydrofolate homocysteine methyltransferase (MTHF-HMT) catalyzes the synthesis of methionine and tetrahydrofolate from homocysteine plus 5-methyltetrahydrofolate. SAH and 5'-methylthioadenosine, produced during polyamine synthesis, may inhibit transmethylation reactions.

Liver-specific *MAT1A* codifies the isozymes MATI and MATIII, which are a tetramer and dimer of the subunit α_1 , respectively.⁴⁴ *MAT2A* codifies an α_2 -subunit, the enzyme MATII isoform, which is widely distributed. *MAT2A* expression prevails in fetal liver and is progressively substituted by *MAT1A* in adult liver.^{43,45} MATI and MATIII isozymes are characterized by intermediate (23 μM –1 mM) and high (215 μM –7 mM) Michaelis constant (K_m) for methionine. As a consequence, the physiological level of liver SAM (~60 μM) poorly inhibits MATI, whereas it stimulates MATIII activity.⁴³ The low K_m (~4–10 μM) for methionine of MATII causes its inhibition by the reaction product.⁴³ A β -subunit of MATII, devoted of catalytic action, is encoded by *MAT2B* gene. This subunit regulates MATII by lowering its K_m for methionine and K_i for SAM.⁴⁴ As a consequence, the association of β and α subunits potentiates MATII inhibition by SAM. Recent observations show a correlation of glycine N-methyltransferase (GNMT) involved in hepatic SAM catabolism (Figure 2), with *MAT1A* and betaine-homocysteine methyltransferase hepatic enzymes, indicating a coordinate regulation of methionine cycle enzymes that determine SAM level.⁴⁵

Various observations indicate that the reconstitution of a normal SAM level and the SAM/SAH ratio results in HCC chemoprevention. Huh7 cell transfectants, stably overexpressing *MAT1A*, exhibit higher SAM content; lower DNA synthesis, proliferative activity, microvessel density, and CD31 and Ki-67 expression; and higher apoptosis than

controls.^{46,47} Further, the administration to rats of highly purified SAM during hepatocarcinogenesis significantly inhibits the development of early preneoplastic liver lesions, dysplastic nodules, HCC, and induces a decrease in labeling index and an increase in apoptosis of preneoplastic cells.^{37–40} It must be noted, however, that SAM infusion for 24 days did not affect the size of already-established tumors in mice transplanted with Huh7 cell transfectants.⁴⁷ It has been hypothesized that a compensatory induction of hepatic GNMT (Figure 2) prevents SAM accumulation, thus hampering a therapeutic effect.

Fall in *MAT1A* expression associated with *MAT2A* up-regulation occurs in liver cirrhosis and rodent and human HCC, leading to a decrease in the *MAT1A*:*MAT2A* ratio (called *MAT1A*/*MAT2A* switch).^{41,48,49} In cirrhotic liver, MATI/III down-regulation, resulting from the oxidation of cysteine residue in the ATP binding site, is associated with GSH fall.^{50,51} Protection of MATI/III and reconstitution of the GSH pool by SAM administration strongly inhibits liver fibrosis both in rats and humans.^{50,52} Limited MATII up-regulation in HCC, because of its inhibition by the reaction product,⁴³ cannot compensate for MATI/III decrease. Therefore, a sharp decrease in MATI/III:MATII activity ratio and an increase in polyamine synthesis (Figure 2) contribute to SAM fall.³⁷ These observations strongly suggest a role in hepatocarcinogenesis of *MAT1A*/*MAT2A* switch and fall in SAM level. Accordingly, *MAT1A*-knockout mice, characterized by chronic SAM deficiency, undergo steatosis and mononuclear cell infiltration in periportal areas.⁵³ HCC develops in a high percentage of these mice at 18 months of age.⁵³

MAT2A up-regulation may also contribute to HCC cell proliferation. In H35 hepatocellular carcinoma cells, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase/v-AKT murine thymoma viral oncogene homolog (PI3K/AKT) pathways are involved in HGF-induced cell proliferation and *MAT2A* up-regulation.⁵⁴ Their inhibition results in proliferation restraint and a decrease in *MAT1A*:*MAT2A* switch.⁵⁴ Moreover, the transfection of *MAT2B* in Huh7 cells that do not express this subunit, followed by the interaction of the β -subunit with the α_2 -subunit, increases DNA synthesis and inhibits SAM production, whereas the down-regulation of β -subunit in HepG2 cells, overexpressing *MAT2B*, inhibits DNA synthesis.⁵⁵

The alterations of methionine metabolism have been comparatively evaluated in c-Myc and c-Myc/transforming growth factor α (TGF- α) transgenic mice to assess their relationships with tumor progression: c-Myc transgenics show lower progression of dysplastic liver to HCC and development of less-aggressive HCC with respect to the double transgenics.⁴¹ Dysplastic nodules and HCC of both transgenic models exhibit a reduction in MATI/

III:MatII activity ratio and *Cbs* (cystathionine beta-synthase) expression, and a rise in *Sahh* expression. This suggests that in these lesions, a relatively high homocysteine production is presumably not associated with an increase in GSH level (Figure 2).⁴¹ In the lesions of both transgenics, *Bhmt* expression decreases, whereas *Mthf-hmt* expression abruptly increases in HCC of Myc/TGF α transgenics and does not change in the lesions of c-Myc transgenics.⁴¹ The expression of genes involved in polyamine synthesis, *Sdc* (SAM decarboxylase), *Odc* (ornithine decarboxylase), *Srs* (spermine synthase), and *Sds* (spermidine synthase), increases in both transgenic mice, with the highest values in the lesions of c-Myc/Tgf- α transgenics (Figure 2). Finally, the expression of the *Mtap1* (5'-methylthioadenosine phosphorylase) gene, encoding a key enzyme of the methionine salvage pathway,⁵⁶ exhibits a significant increase in dysplastic nodules and HCC of Myc/TGF α transgenics, suggesting a rise in methionine resynthesis.⁴¹

Experiments with human HCC showed a similar situation when HCCs with better prognosis (based on survival >3 years after partial liver resection; HCCB) and poorer prognosis (survival <3 years; HCCP), and their corresponding surrounding liver, were examined.⁴¹ In these lesions, *MAT1A:MAT2A* expression ratio and MATI:MATII activity ratio and *BHMT*, *CBS*, and *MTAP1* expression decrease and *SDC*, *ODC*, *SMR*, and *SMS* expression increases with no variation of *SAHH* and *MTHF-HMT* expression in surrounding liver and HCC. These changes are higher in HCC than the surrounding liver and in HCCP with respect to HCCB.⁴¹

The comparative evaluation of the deregulation of methionine metabolism in dysplastic nodules and HCC induced in the Fisher-344 (F344) strain, which is genetically susceptible to hepatocarcinogenesis, and the Brown Norway (BN) genetically resistant strain⁸ showed a lower MATI/III:MATII activity ratio and *Mat1A:Mat2A* expression ratio in fast-growing and progressing F344 rat lesions.⁵⁷ Notably, these ratios were negatively correlated with DNA synthesis and DNA methylation and positively correlated with apoptosis in rat liver lesions.⁵⁷ A positive correlation was found between cell proliferation and global DNA hypomethylation and GI of transgenic mice and human HCC, whereas an inverse correlation of *MAT1A:MAT2A* and MATI/III:MATII ratios with proliferation rate and GI, and a direct correlation with apoptosis and DNA methylation, occurred in human HCC.⁴¹ Finally, prediction of patient's survival length by MATI/III:MATII ratio was shown by Cox analysis.⁴¹

Altogether, these findings indicate a close association of reduced methionine resynthesis, increased in SAM use for polyamine synthesis, and a *MAT1A/MAT2A* switch with HCC progression and prognosis.

Mechanisms of MATs deregulation

Numerous observations associate methylthioadenosine's deregulation with alterations in promoter methylation of MAT genes. *MAT1A* down-regulation in rat cirrhotic liver, induced by chronic CCl₄ treatment, and in the human HepG2 hepatoblastoma cell line has been ascribed to the methylation of CCGG sequences of the *MAT1A* promoter.⁵⁸ CCGG methylation at +10 and +80 of the coding region in Huh7, HEK293, and HepG2 cell lines has been associated with underexpression of *MAT1A*.⁵⁹ Conversely, *MAT2A* overexpression of human HCC was associated with CCGG hypomethylation of gene promoter.⁶⁰ In accordance with these results, recent observations showed the association between *Mat1A/Mat2A* switch and reduced SAM levels with CpG hypermethylation and histone H4 deacetylation of *Mat1A* promoter of fast-growing HCC induced in F344 rats.⁵⁷ In the *Mat2A* promoter, CpG hypomethylation and histone H4 acetylation occurred.⁵⁷ These changes were low or absent in slowly growing HCC of BN rats.⁵⁷ Accordingly, *MAT1A* promoter hypermethylation and *MAT2A* promoter hypomethylation were highest in human HCCP compared with HCCB.⁵⁷ These findings suggest that transcriptional regulation of *MAT1A* and *MAT2A* by promoter methylation may be strongly involved in HCC aggressivity.

Posttranscriptional mechanisms involving mRNA binding proteins may also be implicated in the deregulation of methyl adenosyltransferases. It is known that AUF1 may promote mRNA decay, whereas HuR enhances mRNA stabilization by selective binding to AU-rich elements.^{61,62} Increased HuR and AUF1 protein levels have been found in human livers with HCC.⁶³ Recent work documented a sharp increase of AUF1 and HuR, as well as of MAT1A-AUF1 and MAT2A-HuR ribonucleoproteins, in F344 HCC and human HCCP.⁵⁷ Notably, these changes were very low or absent in slowly progressing HCC of BN rats.⁵⁷

MAT1A under-regulation has also been attributed to up-regulation in HCC of miR-664, miR-485-3p, and miR-495.⁶⁴ Individual knockdown of these miRNAs in Hep3B and HepG2 cells enhanced *MAT1A* expression. The knockdown of miRNAs-664/485-3p/495 increased Hep3B cell tumorigenesis in nude mice, whereas the opposite occurred when these miRNAs were stably overexpressed.⁶⁴

Deregulation of HCC oncosuppressors

The inactivation of oncosuppressor genes plays a crucial role in hepatocarcinogenesis. Numerous studies on oncosuppressor gene deregulation in HCC have shown that aberrant promoter methylation is the mechanism most frequently responsible

for down-regulation of these genes.^{65–68} Variable percentages of HCC show hypermethylation of *APC*, *E-CAD*, *P14^{ARF}*, *P15*, *P16^{INK4}*, *P21^{WAF1}*, *P27^{KIP1}*, *P57^{KIP2}*, *P73*, *P130*, *RAR-B* (retinoic acid receptor, beta), *SOCS-1* (suppressor of cytokine signaling 1), *PRM2* (PR domain-containing protein 2), *GSTP* (glutathione S-transferase), *O6-MDM* (O6-methylguanine-DNA methyltransferase), *RASSF1A* (Ras association domain family 1A), and *FOXO1* (Forkhead box O1). The degree of promoter methylation in HCC is significantly higher than in dysplastic nodules and cirrhotic liver.^{66–68}

P53 gene has been intensively studied in HCC. The *P53* pathway has crucial roles in cell cycle control, transcriptional regulation, and apoptosis.⁶⁹ *P53* deregulation in HCC mostly depends on mutational events.⁶⁹ However, it has been observed that HBx protein binds to the *p53* protein in the cytoplasm, resulting in the blockage of *p53* entry into the nucleus.⁷⁰ Furthermore, the *14-3-3 σ* gene, implicated in G2/M cell cycle arrest by *p53*, is frequently hypermethylated in HCC.⁷¹

Promoter hypermethylation of oncosuppressors is never present in all HCC cases. Recent studies have analyzed some alternative epigenetic mechanisms that could induce oncosuppressor down-regulation. Fast proliferation of dysplastic nodules and HCC requires the inactivation of cell cycle inhibitors. The inhibition of *CDK4* and *CDK6* by *p16^{INK4}* blocks G1 phase by preventing *pRb* phosphorylation. *p16^{INK4}* inactivation in 60%–85% of human HCC depends on promoter GpG methylation.⁷² *p16^{INK4}* inactivation may also be achieved by the linkage of cyclin D1 kinases with *HSP90* (heat shock protein 90) and *CDC37* (cell division cycle 37) proteins,⁷² as well as through the transfer of *E2F4*, a *p16^{INK4}* effector, from nucleus to cytoplasm by *CRM1* (chromosome region maintenance 1).^{73,74} These mechanisms are particularly active in fast-growing dysplastic nodules and HCC of F344 rats.⁷⁵ Moreover, the highest formation of *p16^{INK4}/HSP90/CDC37* complexes was found in human HCCP.⁷⁵

P21^{WAF1}, *P27^{KIP1}*, *P57^{KIP2}*, *P130*, *RASSF1A*, and *FOXO1*, inhibitors of *CYCLIN E*- and *CYCLIN A*-dependent *CDK2*, are down-regulated by promoter methylation in variable percentages of human HCC.⁷⁶ In unmethylated tumors, these inhibitors of G1-S transition are ubiquitinated by the ubiquitin ligase complex *SKP2-CSK1* (S-phase kinase-associated protein 2–Cdc28 protein kinase 1) ligase, followed by proteasomal degradation.⁷⁶ *CDK2* inhibitors are more frequently down-regulated in HCCP than in HCCB, either by promoter hypermethylation or proteasomal degradation.⁷⁶ These observations and the existence of a positive correlation between *SKP2* expression and HCC cell proliferation

and microvascularization, and a negative correlation with apoptosis,⁷⁶ suggest a connection of the degradation of *CDK2* inhibitors with HCC aggressivity. Researches on rat liver carcinogenesis showed higher ubiquitination of *CDK2* inhibitors by *Skp2–Cks1* in fast-growing dysplastic nodules and HCC of genetically susceptible F344 rats than in slow-growing lesions of resistant BN rats,⁷⁷ thus suggesting a genetic control of cell cycle inhibitor inactivation by proteasomal degradation.

SKP2 activity is regulated by the *SKP2* suppressor *HINT1* (histidine triad nucleotide binding protein 1) and *CDC14B* (cell division cycle 14, *Saccharomyces cerevisiae* homolog B) phosphatase, which induces *SKP2* dephosphorylation, followed by degradation mediated by the ubiquitin ligase (*APC/C*) *CDH1* (anaphase-promoting complex/cyclosome and its activator *CDH1*).⁷⁸ The investigation of the interstrain differences in the degradation of *CDK2* inhibitors indicated the involvement of the up-regulation of *HINT1* and *CDC14B*.⁷⁹ Interestingly, the mechanisms blocking *SKP2* degradation, such as down-regulation of *CDC14B*, associated with *CDK2*-dependent serine phosphorylation (which impedes the interaction between *CDH1* and *SKP2*), and *HINT1* inactivation, are much higher in HCCP than in HCCB.⁷⁷

Among the genes involved in methionine and SAM metabolism, *GNMT* is underexpressed both in rat and human HCC, with the lowest expression in fast-growing F344 HCC and human HCCP with respect to slowly growing BN HCC and HCCB.⁸⁰ Remarkably, loss of *GNMT* was found to induce liver steatosis and HCC.⁸¹ Recent work indicates that DNA hypermethylation plays an important role in *GNMT* suppression in HCC.⁸² Loss of *GNMT* causes SAM accumulation.⁷⁴ Histone and genome-wide methylation analyses suggest that *GNMT* deregulation in HCC could be involved in promoter-specific DNA methylation of oncosuppressor genes.⁸²

DNA methylation status of liver cancer stem cells

An emerging aspect of the studies on the role of epigenetic alterations in carcinogenesis is the possibility that these alterations are involved in the regulation of cancer stem/progenitor cells.⁸³ Side population cells, possessing typical cancer stem/progenitor cell-like properties, isolated from HCC cell lines Huh7 and PLC/PRF/5, induce HCC in NOD/SCID mice.⁸³ Genome-wide DNA methylation microarray analysis showed the presence, in the two cell lines compared with their corresponding non-side population cells, of both hypermethylated and hypomethylated genes, which were related to four and eight pathways, respectively.⁸⁴

In keeping with these observations, recent studies revealed altered methylation status of the promoter of *OCT4*, *SOX2*, *KLF4*, and *c-MYC* genes in HCC.⁸⁵ The overexpression of these genes induces pluripotency in somatic cells, leading to the generation of embryonic stem cell-like pluripotent cells.^{86,87}

Compared with normal liver cells L02, which displayed a modest methylation level, DNA hypomethylation of *NANOG* was found in the HCC cell lines PLC (primary HCC) and MHCC97L and MHCC97H, derived from metastatic HCC, with the lowest decrease in DNA methylation in the low metastatic MHCC97L cells.⁸⁵ A reduced methylation pattern of *OCT4* promoter and *c-MYC* exon 3 was also observed in PLC and MHCC97L cells. These changes were associated with *NANOG*, *OCT4*, and *c-MYC* up-regulation. *NANOG* promoter hypomethylation and gene up-regulation were also observed in human HCC primary tumor tissue.⁸⁵ Importantly, a cross-regulation between *OCT4* and *NANOG* in cancer cells by reprogramming of promoter methylation was demonstrated, suggesting a role of epigenetic reprogramming of gene expression in the acquisition of stem cell-like properties.⁸⁵ This conclusion is enforced by recent observations indicating a role of DNMT1 depletion in the enhancement of cancer stem cell properties in WRL68 HCC and HepG2 hepatoblastoma cell lines.⁸⁸

These observations suggest that DNA methylation status is a key epigenetic mechanism that determines the pool of cancer stem cells in liver cancer by maintaining the pluripotency and regulating the differentiation of stem cells during cancer progression.

Signaling deregulation

Role of altered methionine metabolism

The role of *MAT1A/MAT2A* switch and SAM fall in HCC has been evaluated by studying the effect of reconstituting *MAT1A* expression and SAM level on signal transduction. Forced expression of *MAT1A* in the Huh7 and HepG2 cell lines down-regulates *cyclin D1*, *E2F1*, *IKK*, *NF- κ B*, and antiapoptotic *BCL2* and *XIAP* genes and up-regulates proapoptotic *BAK* and *BAX* genes.^{57,89} Nuclear factor κ B activation in rat preneoplastic foci is inhibited by SAM.⁹⁰ The latter also restrains AMP activated protein kinase (AMPK), phosphorylated AKT (pAKT), and phosphorylated extracellular signal-regulated protein kinase (pERK) activities by up-regulating the oncosuppressor *PP2A*.^{91,92} SAM levels and *PP2A* expression are inversely correlated with pAKT and pERK expression both in rat and human HCC.^{91,92} Recent research aimed at investigating the mechanism of a SAM inhibitory effect on ERK1/2 activity showed a possible interference of SAM with the activity of DUSP1, a specific

ERK inhibitor. HCC of F344 rats and human HCC exhibit low DUSP1 expression associated with ERK1/2 up-regulation.^{93,94} DUSP1 phosphorylation at Ser296 by active ERK1/2 leads to its ubiquitination by SKP2-CKS1 ubiquitin ligase, followed by proteasomal degradation.^{93,94} In addition, ERK1/2 supports SKP2-CKS1 activity through the activation of FOXM1.⁹⁵ Recent work discovered a reduction of *DUSP1* mRNA and, at a higher extent, of DUSP1 protein levels in the livers of *MAT1A*-KO mice and in in vitro cultures of mouse and human hepatocytes.⁹⁶ SAM treatment increased DUSP1 mRNA and protein levels, suggesting a regulation of DUSP1 expression at transcriptional and posttranslational levels.⁹⁶ Interestingly, hypoxia could also be involved in SAM deregulation of ERK1/2 expression. It is known that the tumor necrosis factor (TNF)- α /HIF-1 α axis sustains the expression of *FOXM1*,⁹⁷ which is a mediator of *ERK1/2* effects on cell proliferation.⁹⁵ The observation that HIF-1 α can bind *MAT2A* promoter suggests the possibility that *ERK1/2* up-regulation in BEL-7404 and Hep3B HCC cells and HepG2 cells could be at least partially attributed to a reduction of SAM level during hypoxia.⁹⁸

Recent work⁹⁹ showed that *Apex1* mRNA and protein levels are significantly reduced in the liver of *MAT1A*-KO mice. *Apex1* expression was correlated to the increase of AP sites and the reduction of the expression of *Apex1* targets *Bax*, *Fas*, and *p21*.⁹⁹ An association of *MAT1A* mRNA decrease with *APEX1* and *c-MYC* mRNAs increase was found in cell cultures of human and mouse hepatocytes in which, however, *APEX1* protein level decreased to 60% of baseline.⁹⁹ The prevention of these changes by SAM was attributed to possible proteasome inhibition.¹⁰⁰

Signaling regulation by miRNA

Different studies on experimental models have ascertained the deregulation of miRNAs even in early stages of liver carcinogenesis. Up-regulation of miR-17-92 cluster, miR-106a, and miR-34 was observed during tamoxifen-induced hepatocarcinogenesis in female rats.¹⁰¹ Thirty differential expressed miRNAs have been identified by microarray analysis of mice fed a MDD.¹⁰² Down-regulation of miR-15/16, miR-26a, miR-34a, miR-150, and miR-195 occurs in c-Myc-transgenic mice.¹⁰³

Numerous studies showed the enhancement of cell cycle and signal transduction in HCC induced by up-regulation of miRNAs targeting oncosuppressor or apoptogenic genes (Table 1). MiR-423¹⁰⁴ and miR-221¹⁰⁵ target wildtype p53-activated fragment (WAF) and cyclin-dependent kinase inhibitor (KIP) family inhibitors of cell cycle, respectively. MiR-602 up-regulation may enhance RAS/ERK and MSP/YAP

Table 1 Targets and functional effects of microRNAs up-regulated in hepatocarcinogenesis

microRNA	Targets	Functional effects*	Reference
miR-423	P21/WAF1	Enhanced CDK2 activity (G1-S transition)	104
miR-221	P27/KIP1, p57/KIP2	Enhanced CDK2 activity (G1-S transition)	105
miR-93, miR-106b	E2F1	Prevention of excessive E2F1 accumulation (?)	112
miR-602	RASSF1A	Enhanced RAS/ERK, MST/YAP, pathways, JUN	106
miR-222	PP2A	Enhanced AKT pathway	107
miR-221, miR-222, miR-21	PTEN	Enhanced AKT pathway	108
miR-221	DDIT4	TSC1/2 complex inhibition and AKT activation	109
miR-221, miR-222 miR-181b/d	TIMP3	Enhanced MMP2 and MMP9	110
miR-221	BMF	Apoptosis inhibition	111
miR-106b, miR-93, miR-25	BIM	Apoptosis inhibition	112
miR-221, miR-222	TRAIL-induced apoptosis	Inhibition of caspases 3, 6, 7, 8	109
miR-192	ERCC3, ERCC4	Inhibition of DNA excision repair	115

Note: *Functional effects of miRNA up- or down-regulation.

Abbreviations: AKT, v-AKT murine thymoma viral oncogene homolog; BIM, BCL2 interacting protein; BMF, BCL2 modifying factor; CDK2, cyclin-dependent kinase 2; DDIT4, DNA damage-inducible transcript 4; E2F1, E2F transcription factor 1; ERCC3/4, excision-repair, complementing defective, in Chinese hamster, 3/4; ERK, extracellular signal regulated kinase; JUN, v-JUN avian sarcoma virus 17 oncogene homolog; KIP 1, cyclin-dependent kinase inhibitor 1; MMP, matrix metalloproteinase; MST, homologues of Hpo; PP2A, Protein phosphatase 2A; PTEN, Phosphatase and tensin homologue deleted on chromosome 10; RAS, rat sarcoma; RASSF1A, Ras association domain family 1A; TIMP3, tissue inhibitor of metalloproteinase 3; TRAIL, TNF-related apoptosis-inducing ligand; YAP, Yes kinase-associated protein.

pathways through RASSF1A inhibition.¹⁰⁶ *PP2A* and/or *PTEN* inhibition results from up-regulation of miR-221, miR-222, and miR-21.^{107,108} Furthermore, miR-221 overexpression inhibits *DDIT4* (DNA damage-inducible transcript 4), a regulator of the mammalian target of rapamycin (mTOR) kinase, by enhancing the activity of the putative oncosuppressor TSC1/2 (tuberous sclerosis 1/2) complex.¹⁰⁹ Liver cell migration and metastasis may be favored by miR-221, miR-222, and miR-181b/d overexpression that enhances *MMP2* and *MMP9* (matrix metalloproteinase 2 and 9) activity through *TIMP3* (tissue inhibitor of metalloproteinase 3) inhibition¹¹⁰ (Table 1). Finally, a miR-221 and miR-106b-25 cluster, containing miR-106b, miR-93, and miR-25, may modulate apoptosis by targeting the proapoptotic genes *BMF* and *BIM* genes^{111,112} (Table 1).

Surprisingly, up-regulation of miR-93 and miR-106b, targeting *E2F1*, a key gene of G1-S transition, was observed in HCC.¹¹² It has been hypothesized¹¹³ that miR-93 and miR-106b up-regulation hinders the accumulation of high E2F1 levels that could induce apoptosis.¹¹⁴

A contribution of miRNA deregulation to DNA damage accumulation in HCC has been suggested on the basis of the discovery of miR-192 up-regulation, leading to inhibition nucleotide excision repair by targeting *ERCC3* and *ERCC4* (excision-repair, complementing defective, in Chinese hamster 3 and 4) in the HBV-related HCC cell line HepG2.2.15¹¹⁵ (Table 1).

Several miRNAs exhibit an oncosuppressor activity, and their down-regulation in HCC enhances signal transduction and growth (Table 2). Activation of cell cycle may result from the down-regulation of miR-26a,¹¹⁶ targeting *Cyclins D2* and *E2* genes; of miR-195,¹¹⁷ targeting *Cyclin D1*, *CDK6*, and

E2F3; and of miR-122a,¹¹⁸ which targets *Cyclin G1*. miR-122 underexpression is also associated with overexpression of *PBF* (pituitary tumor-transforming gene 1 binding factor), which promotes tumor growth.¹¹⁹ G2-M transition may be favored by *STMN1* (Stathmin) overexpression and microtubules stabilization¹²⁰ induced by miR-223 down-regulation.

Numerous observations attribute oncosuppressor activity to Let-7 miRNA family (Table 2). The underexpression of hsa-Let-7g, a positive regulator of c-*MYC* and a negative regulator of *p16^{INK4A}*,¹²¹ is higher in metastatic HCC than in metastasis-free HCC.¹²² The deregulation of collagen synthesis and the enhancement of epithelial- mesenchymal transition (EMT) are involved in the prometastatic effect of Let-7g down-regulation. Let-7g targets *COL1A2* (type I collagen a2), and cell migration is suppressed in Let-7g overexpressing huH1-let-7g cells. This latter effect is eliminated by the addition to the cell culture of type I collagen a2.¹²²

Stimulation of HCC cell migration and EMT transition follows the activation of *ZEB1/2* (zinc finger e box-binding homeobox 1/2) by down-regulation of miR-200b¹²³ and of *ROCK2* (RHO-associated coiled-coil-containing protein kinase 2) and *EZH2* (enhancer of ZESTE, drosophila, homolog 2) by down-regulation of miR-124.¹²⁴ Furthermore, miR-194 overexpression suppresses *N-cadherin* expression, thus inhibiting the migration of mesenchymal-like cancer cells.¹²⁵ Accordingly, miR-148a forced expression in MHCC97-H cells inhibits the expression of *SNAIL* and *N-cadherin*, suggesting EMT suppression.^{126,127} Migration of human HCC cells is also suppressed by miR-23b targeting *uPA* and c-*MET*¹²⁸ (Table 2).

Table 2 Targets and functional effects of microRNAs down-regulated in hepatocarcinogenesis

microRNA	Targets	Functional effects*	Reference
miR26a	Cycl D2, E2, IL6	Enhanced G1-S transition	26, 116
miR195	Cycl D1, CDK6, E2F3	Enhanced G1-S transition	117
miR-122	Cycl G1	Enhanced cell cycle progression	118
	PBF	Stimulation of HCC proliferation	119
miR-223	STMNI	Microtubules stabilization (G1-M transition)	120
Let-7G	Collagen I- α 2	Enhanced cell migration	122
miR-200b	ZEB1, ZEB2	Stimulation of EMT	123
miR-124	ROCK2, EZH2	Stimulation of EMT	124
miR-194	N-Cadherin	Stimulation of EMT	125
miR-148a	SNAIL, N-Cadherin	Stimulation of EMT	126,127
miR-23b	uPA, c-MET	HCC cell migration	128
miR-139	ROCK2, c-FOS, TCF-4	HCC cell migration	129,130, 132
miR-99a	IGF-1R, mTOR	Stimulation of HCC proliferation	133
miR-122	NDRG3	Stimulation of HCC proliferation	134
miR-375	AEG-1	Stimulation of HCC proliferation	135
	YAP	Stimulation of HCC proliferation	136
miR-338-3p	SMO	Stimulation of HCC proliferation	137
miR34a	c-MET	Stimulation of HCC proliferation	138
miR-203	SURVIVIN	Stimulation of HCC proliferation	139
miR-124	PIK3CA	Enhanced PI3K/AKT pathway	140
	STAT3	Enhanced STAT3 signaling	141
miR-637	STAT3	Enhanced STAT3 signaling	142
miR-491-5p	AFP, HSP90, NF-kB	Inhibition of TNF- α -related apoptosis	145
miR-145	HDAC2	Hepatocarcinogenesis promotion	143
	ADAM17	HCC metastases	146
miR-214	FGF-R1	HCC metastases	147
miR-22	HDAC4	Hepatocarcinogenesis promotion	144
miR-22 and miR-140-3-p	NCOA1, NRIP1	Apoptosis inhibition	152
miR-122	BCL-W	Apoptosis inhibition	153
miR-29	BCL-2, MCL-1	Apoptosis inhibition	154
miR-101	MCL-1	Apoptosis inhibition	155
	DNMT3A	Enhanced methylation of oncosuppressors	157
miR-29c	TNFAIP3	Apoptosis inhibition	156

(Continued)

Table 2 (Continued)

microRNA	Targets	Functional effects*	Reference
miR-152	DNMT1	Hypermethylation of GSTP1, CDH1	158

Note: *Functional effects of miRNA up- or down-regulation.

Abbreviations: ADAM17, a disintegrin and metalloproteinase domain 17; AEG-1, astrocyte elevated gene-1; AFP, alpha-fetoprotein; AKT, v-AKT murine thymoma viral oncogene homolog; BCL-2, B-CELL CLL/Lymphoma 2; BCL-W, BCL-like 2; CDH1, cadherin 1; CDK6, cyclin-dependent kinase 6; c-FOS, FBJ murine osteosarcoma viral oncogene homolog; c-MET, hepatocyte growth factor receptor; Cycl D1, D2, E, G1, Cyclin D1, D2, E, G1; DNMT1, 3A, DNA methyltransferase 1, 3A; E2F3, E2F transcription factor 3; EMT, epithelial-mesenchymal transition; EZH2, enhancer of ZESTE, drosophila, homolog 2; FGF-R1, fibroblast growth factor, receptor 1; GSTP1, glutathione S-transferase P1; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HSP90, heat shock protein 90; IGF-1R, Insulin-like growth factor, receptor 1; IL6, interleukin 6; MCL-1, myeloid cell leukemia sequence 1; mTOR, mammalian target of rapamycin; NCOA1, nuclear receptor coactivator 1; NDRG3, NMYC downstream-regulated gene 3; NF-kB, nuclear factor kB; NRIP1, nuclear receptor-interacting protein 1; PBF, pituitary tumor-transforming gene binding factor; PI3K, phosphatidylinositol 3 kinase; PIK3CA, phosphatidylinositol 3-kinase, catalytic, alpha; ROCK2, RHO-associated coiled-coil-containing protein kinase 2; SMO, smoothened; STAT3, signal transducer and activator of transcription 3; STMNI, stathmin 1; TCF-4, T cell factor 4; TNFAIP3, tumor necrosis factor alpha-induced protein 3; uPA, plasminogen activator, urinary; YAP, yes associated protein 1; ZEB1/2, zinc finger E box-binding homeobox 1/2.

A crucial role in HCC cell migration is also played by miR-139. MiR-139 down-regulation in HCC is significantly correlated to the poor prognosis and metastatic potential of this tumor.^{129,130} Forced overexpression of miR-139 in human HCC cell lines SMMC-7721 and BEL7402 significantly inhibits cell migration and invasion in vitro, as well as tumor incidence and lung metastasis in mouse orthotopic transplants of HCC. Mechanistic studies demonstrated that miR-139 interacts with the 3'-UTR of *ROCK2* and inhibits its expression in HCC cells.¹²⁹ These observations are consistent with the known capacity of the Rho-kinases to activate the actomyosin contraction, cellular migration, and chemotaxis.¹³¹

Additional mechanisms of the prometastatic effect of miR-139 down-regulation in HCC have been recently discovered. Evidence has been presented in favor of a contribution of increased *c-FOS* expression, associated with miR-139 down-regulation, to the high metastatic potential of human HCC cell sublines.¹³⁰ Furthermore, it has been observed that the inhibition of HCC cell proliferation and invasion, induced by miR-139 forced overexpression, is associated with suppression of TCF-4 (T-cell factor 4) activity, suggesting a contribution of miR-139 down-regulation to the increase in β -catenin/TCF-4 transcriptional activity in HCC.¹³²

Stimulation of HCC cell proliferation also occurs by down-regulation of miR-99a,¹³³ miR-122,¹³⁴ miR-375,^{135,136} miR-338-3p,¹³⁷ miR-34a,¹³⁸ and miR-203¹³⁹ that target *IGF-1R/mTOR*, *NDRG3* (N-myc downstream-regulated gene 3), *AEG-1* (astrocyte elevated gene-1)/*YAP* (Hippo signaling), Hedgehog signaling, *c-MET*, and *SURVIVIN*, respectively

(Table 2). Interestingly, targeting of *c-MET* by miR-34a induces a decrease in c-MET-induced phosphorylation of ERK1/2.¹³⁸ Inhibition of miR-124 and miR-637, leading to enhanced PI3K/AKT¹⁴⁰ and/or STAT3^{141,142} signaling, may result in increased hepatocarcinogenesis.¹⁴³ Promotion of liver cancer may also result from up-regulation of *histone deacetylase 2 (HDAC2)* and *HDAC4* targeted by miR-145¹⁴³ and miR-22,¹⁴⁴ respectively, HCC development is also facilitated by the under-regulation of miR-491-5p, targeting *AFP*, *HSP90*, and *NF- κ B*, through inhibition of TNF- α -induced apoptosis.¹⁴⁵ Interestingly, recent observations indicate that miR-145 represses HCC cell invasion by targeting *ADAM17* (a disintegrin and metalloprotease 17).¹⁴⁶ Notably, *ADAM17* is also targeted by miR-122, the down-regulation of which induces HCC metastasis.¹⁴⁷ HCC metastasis is also stimulated by miR-214 down-regulation, leading to *FGFR-1* (fibroblast growth factor receptor 1) gene overexpression and HCC progression.¹⁴⁸

In HBV-related hepatocarcinogenesis, miR-122 expression is inversely correlated to NDRG3 protein level¹³⁴ (Table 2). The forced expression of miR-122 into the HBV-related HepG2.2.15 cell line suppresses the expression of *NDRG3* (n-myc downstream-regulated gene 3), with subsequent reversion of the malignant phenotype. The restoration of miR-122 inhibits HBV replication and HCC cells proliferation.

Numerous other miRNAs exhibit a prometastatic potential. Among these, miR-550a, up-regulated in HCC, inhibits *CPRB4* (cytoplasmic polyadenylation element binding protein 4) expression, which impedes HCC cell migration and invasion;¹⁴⁹ miR-210, induced by hypoxia, increases the metastatic potential of HCC by targeting *VMP1* (vacuole membrane protein 1);¹⁵⁰ and targeting of *GNAI2* (G protein, alpha-inhibiting 2B) by miR-30d induces HCC cell invasion and metastasis.¹⁵¹

MiRNAs down-regulation may also contribute to hepatocarcinogenesis by increasing cancer cell survival (Table 2). miR-22 and miR-140-3-p down-regulation stimulates antiapoptotic signaling in HCC by targeting *NCOA1* (nuclear receptor coactivator 1) and *NRIP1* (nuclear receptor-interacting protein 1),¹⁵² miR-122 down-regulation inhibits the antiapoptotic gene *BCL-w*,¹⁵³ and miR-29 down-regulation favors the expression of the antiapoptotic genes *BCL-2* and *MCL-1*.¹⁵⁴ *MCL-1* is also targeted by miR-101 that is down-regulated in HBV-related HCC.¹⁵⁵ Interestingly, according to recent observations, forced overexpression of miR-29c in HepG2.2.15 cells leads to inhibition of *TNFAIP3* (TNF alpha-induced protein 3) expression, HBV DNA replication, and cell proliferation, associated with increase in cell death.¹⁵⁶

The down-regulation of miR-101 by the HBx protein also induces aberrant DNA methylation by targeting *DNMT3a*.¹⁵⁷ In HBV-related HCC, aberrant DNA methylation may likewise occur by down-regulation of miR-152, the expression of which is inversely correlated to *DNMT1* mRNA.¹⁵⁹ Inhibition of miR-152 expression in liver cell lines causes global DNA hypermethylation and increased methylation of oncosuppressors *GSTP1* (glutathione S-transferase pi 1) and *CDH1* (E-cadherin 1)¹⁵⁸ (Table 2).

The induction of miR-23a-27a-24 cluster expression by TGF- β in Huh-7 cells attenuates the antiproliferative and proapoptotic effects of TGF- β .¹⁵⁹ miR-23a-27a-24 cluster is overexpressed in HCC cells and promotes their proliferation and survival.¹⁶⁰ Furthermore, in mice fed a MDD, TGF- β increases miR-181b expression, resulting in *TIMP3* inhibition and stimulation of *MMP2* and *MMP9* (Table 1), with consequent enhancement of HCC cell proliferation and tumorigenicity.¹¹⁰ These findings, taken altogether, suggest that induction of specific miRNAs by TGF- β may abolish its tumor-suppressive effect for HCC.

Recent observations indicate a possible role of miRNAs as prognostic markers. miR-15b, miR-21, miR-130b, and miR-183 are highly expressed in HCC. A recent cross-center validation study demonstrated that combined miR-15b and miR-130b serum levels are a classifier useful to identify early-stage HCCs not detected by serum α -fetoprotein.¹⁶¹ Furthermore, a cluster of 23 miRNAs arising from imprinted genomic loci and associated with poor clinical outcomes was identified.¹⁶² This cluster is encoded within the *Dlk1-Gtl2* imprinted region on mouse chromosome 12qF1 and the human *DLK1-DIO3* region on chromosome 14q32.2. *DLK1*-miRNA cluster is overexpressed in HCC of *c-MET* transgenic mice and human HCC. Interestingly *DLK1*-miRNA cluster up-regulation in human HCC is positively correlated with HCC stem cell markers, such as *CD133*, *CD90*, *EpCAM*, and *Nestin*.⁶⁴

These observations indicate that a complex miRNAs deregulation is strongly involved in hepatocarcinogenesis. The analysis of the mechanisms leading to miRNA deregulation has shown frequent amplification of the 8q24.3 chromosomal region encoding miR-151¹⁶³ and frequent loss of 18q21.31 region encoding miR-122 in HCC.¹⁶⁴ Noticeably, various polymorphisms of *Dicer1* and *RAN* (Ras-related nuclear protein) that, in association with GTP acts as a cofactor of exportin 5 (Figure 1), occur in HCC.¹⁶⁵ The association of these polymorphisms with cancer susceptibility and survival has been reported. *DICER1* rs1057035 CT/CC variant genotypes are associated with decreased HCC risk compared with wild-type TT. This behavior seems to depend on a rise

in affinity binding to hsa-miR-574-3p, which results in a positive feedback of hsa-miR-574-3p.¹⁶⁶

Increased susceptibility to HCC has been related to pri-miR-146a,¹⁶⁷ miRNA-196a2,^{168,169} pri-miR-218,¹⁷⁰ and miR-34b/c.¹⁷¹ miR-122 binds to the *IL1A* (interleukin 1 alpha) gene and is abolished by the insertion of TTCA sequence in the in *IL1A* 3'-UTR region.¹⁷² As a consequence, *IL1A* is overexpressed, likely increasing susceptibility to HCC.¹⁷² Interestingly, according to a recent report, the overexpression of miR-25-93-106b cluster suppresses the expression of *MICA* (MHC class I polypeptide-related sequence A) gene.¹⁷³ The activity of this gene has been associated with the risk for hepatitis-virus-related HCC.¹⁷³ These findings seem to connect miRNA deregulation to the susceptibility to HCC, a topic that deserves further attention.

Numerous observations indicate an epigenetic deregulation of miRNAs expression in different tumors, including HCC. MiR-1 down-regulation in human HCC is associated with promoter methylation.¹⁷⁴ Treatment of primary HCC cultures with the hypomethylating agent 5-aza-2'-deoxycytidine caused miR-1 reexpression, down-regulation of miR-1 targets, including *FoxP1*, *MET*, and *HDAC4*, overexpressed in HCCs, and inhibition of cell cycle progression and cell survival.¹⁷⁴

Epigenetic repression by promoter methylation has been demonstrated for miRNAs exhibiting oncosuppressor activity, such as miR-129-2,¹⁷⁵ miR-132,¹⁷⁶ miR-335,¹⁷⁷ miR-24, and miR-203.¹⁷⁸ According to recent observations, HBx is able to inhibit miR-205, a potential tumor-suppressor gene, by inducing hypermethylation of its promoter.¹⁷⁹

Notably, hypomethylation of the *hsa-miR-191* locus causes miR-191 overexpression and promotes EMT in HCC.¹⁸⁰ Recent observations showed up-regulation of *HDAC1-3* in HCC.¹⁸¹ Their inhibition by trichostatin A in HCC cell lines resulted in increased levels of hsa-miR-449a, leading to reduced *c-MET* mRNA level. *HDAC1-3* overexpression, by causing miR-449a down-regulation, leads to *c-MET* overexpression that favors HCC cell growth.¹⁸¹ These observations assign to chromatin-modifying drugs, including HDAC inhibitors, a potential therapeutic role for HCC.¹⁸²

Gene interactions are also involved in miRNA deregulation. *RACK1* (receptor for activated protein kinase C), frequently underexpressed in HCC, interacts with KSRP (KH-type splicing regulatory protein), a member of DICER¹⁸³ (Figure 1). KSRP binds to and promotes the maturation of miRNA precursors¹⁸⁴ and is involved in recruiting mature miRNAs to RISC¹⁸³ (Figure 1). These findings attribute to a decrease in *RACK1*; in HCC, a decrease in miRNAs function.

Two oncogenes overexpressed in HCC,¹⁸⁵ *SND1* (Staphylococcal nuclease domain-containing 1) and *AEG1* (also called Metadherin), are components of RISC. RISC activity enables the degradation of oncosuppressor mRNAs by the oncogenic miR-221 (Table 1), thus promoting hepatocarcinogenesis.¹⁸⁵ One of the mechanisms involved in miR-221-induced oncogenesis is the up-regulation of angiogenin and CXCL16.¹⁸⁶ Up-regulation of miR-221 by AEG1 or SND1 also leads to inhibition of *PTEN* and *P57* (CDKN1C). *SND1* and *AEG1* also up-regulate various miRNAs targeting oncosuppressors, such as miR-21, which targets *PTEN* and *SPRY2* (Sprouty 2); miR-106b, which targets *P21* (CDKN1A); and miR-93-17,18, which targets *TGFBR2*.¹⁸⁶

The role of SND1 in tumorigenesis is strengthened by the observation that human SND1 protein binds PIWIL1 (HIWI), a member of the PIWI protein family that is implicated in RNA silencing.¹⁸⁷ Up-regulation of *PIWIL1* gene occurs in various cancer types, including HCC.¹⁸⁸ Recent observations showed up-regulation of HIWI protein and mRNA in the metastatic HCC cell lines HCCLM3, MHCC97H, and MHCC97L, the proliferation and invasiveness of which decreased after HIWI depletion.¹⁸⁹ Positive expression of HIWI was associated with larger tumor size and presence of intrahepatic metastases and was an independent risk factor for overall survival and recurrence-free survival.¹⁸⁹

Importantly, the presence of PIWI proteins and Piwi-interacting RNAs is thought to result in aberrant DNA methylation and silencing of genomic regions and to be critical for the maintenance of genomic integrity in germline stem cells.¹⁹⁰ Work from different groups suggests the presence in cancer cells of epigenetic and signaling pathways common to stem cells.¹⁹¹ The HIWI-mediated methylation results in DNA hypermethylation with subsequent genetic and epigenetic changes that recapitulate the aberrant "stem-like" status, allowing cells to possess the self-renewal capacity of stem cells, which characterizes tumorigenesis. Accordingly, it has been found that HIWI increases DNA methylation levels and consequent silencing of cyclin-dependent kinase inhibitor (CDKI).¹⁸⁸

Finally, recent findings indicate that the binding of the PPAR γ (peroxisome proliferator activated receptor-gamma) and RXR α (retinoid X receptor alpha) complex to the DR1 and DR2 consensus site of *miR-122* gene promoter enhances *miR-122* gene transcription.¹⁹² HBx protein binding to PPAR γ inhibits *miR-122* transcription. This epigenetic mechanism is absent in HCV-related HCC, in which other mechanisms, not yet known, cause miR-122 down-regulation.¹⁹²

Therapeutic perspectives

The existence in HCC of a global hypomethylation of DNA, associated with promoter hypermethylation of tumor suppressor genes, encouraged the evaluation of therapies aimed at correcting the alterations of DNA methylation. The attempt to contrast global DNA hypomethylation with SAM administration did not exhibit any therapeutic effect.⁴⁷ However, a well-documented chemoprevention of HCC by SAM, administered during experimental hepatocarcinogenesis, was reported.^{38–40,47} Furthermore, the observation of a SAM curative effect on alcoholic steatohepatitis and hepatitis C^{193–196} suggests a possible chemoprevention of some types of human HCC. SAM chemoprevention of hepatitis C is under evaluation by a current phase II clinical trial.¹⁹³ However, silencing of *MAT2A* or *MAT2B* in HepG2 cells inhibits the proliferative response to leptin.¹⁹⁷ The intracellular transduction of viral vectors in vivo still presents numerous restrictions.¹⁹⁸ Recent observations on the curative effect for HCC of a family of fluorinated N,N-dialkylaminostilbenes are particularly important in this context. Binding of these compounds to MATII α catalytic subunit inhibits SAM synthesis and in vitro and in vivo proliferation of colorectal cancer cells.¹⁹⁹ Moreover, the observation that N,N-dialkylaminostilbenes inhibit the WNT/ β -catenin pathway suggests they could be particularly effective against β -catenin mutated HCC.¹⁹⁹

The promoter hypermethylation of oncosuppressor genes, in variable percentages of HCC, leads to the suppression of their expression.^{198,200,201} This is associated with *HDAC* overexpression, an epigenetic alteration that contributes to the development and maintenance of HCC.²⁰² These findings suggest the possibility of new therapeutic approaches based on modulation of the methylation status of oncosuppressors and the acetylation pattern of histones associated with DNA. Promising results in the treatment of HCC have been obtained by hydroxamate-based histone deacetylase Inhibitors, which synergize with other anticancer agents, such as cytostatic compounds.^{203,204} Moreover, studies on HCC cell lines and xenografted HCC clearly showed a significant increment of the inhibition of HCC cell growth in vivo by the association of HDAC inhibitors with the demethylating agent 5-aza-2'-deoxycytidine.²⁰⁵ Interestingly, according to recent research, 5-aza-2'-deoxycytidine down-regulates the telomerase reverse transcriptase (hTERT) and represses telomerase activity in SMMC-7721 and HepG2 cell lines.²⁰⁴ This is associated with the reversion of the methylation status of hTERT promoter in SMMC-7721, but not in HepG2 cells, suggesting

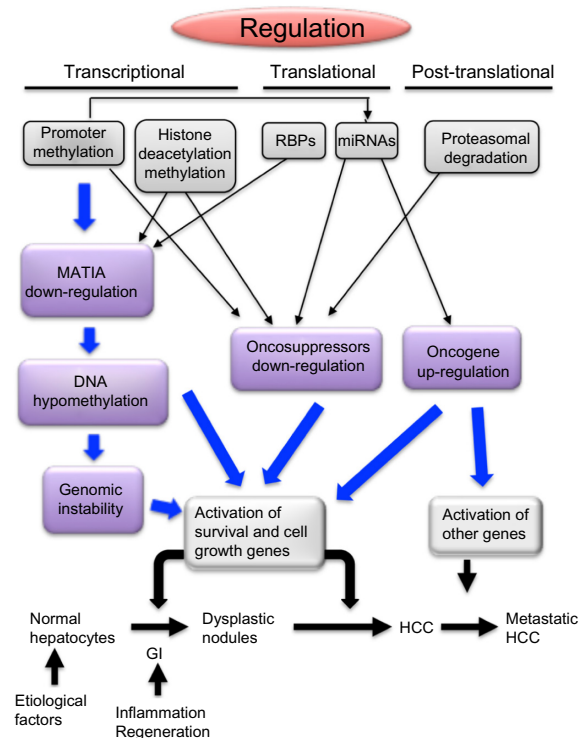


Figure 3 Transcriptional, translational, and posttranslational mechanisms leading to deregulation of *MAT1A*, tumor suppressor genes, and oncogenes during hepatocarcinogenesis.

Abbreviations: miRNAs, microRNAs; *MAT1A*, methionine adenosyltransferase IA; HCC, hepatocellular carcinoma; GI, genomic instability.

that hTERT gene expression is not exclusively regulated by promoter methylation in HCC cells treated with 5-aza-2'-deoxycytidine.²⁰⁴

The observation that the overexpression of several miRNAs is involved in hepatocarcinogenesis suggests a possible therapeutic effect of specific anti-miRNAs oligonucleotides. However, miRNA-based therapeutic methods achieving real inhibition and safety in vivo are still lacking. Recently, linear DNA sequences expressing anti-HBV micro-RNA shuttles were shown to efficiently silence HBV markers in cell culture and in vivo.²⁰⁶ Inhibition of HBV replication and HCC growth was obtained by a lentiviral miRNA-based system.²⁰⁷ Further, the systemic administration in a mouse HCC of an adeno-associated virus preparation of miR-26A, underexpressed in this tumor, was found to significantly inhibit cancer cell survival, proliferation, and progression in the absence of toxicity. Harmless and efficacious delivery of anti-miRNAs oligonucleotides waits for novel modification, conjugation, or formulation strategies. Different studies tested the antitumor effect of different miRNA inhibitors, and promising results were obtained in recent clinical trials with 15 nucleotide antisense oligonucleotides and 8 nucleotide versions in nonhuman primates.^{208,209} Finally, miRNAs were shown to

enhance the effect of anticancer drugs such as doxorubicin and sorafenib.^{210–212}

Conclusion

Numerous chromosome aberrations and oncogene and oncosuppressor mutations have been discovered in HCC.^{2,213–215} Polymorphisms of genes involved in miRNA maturation, such as *DICER1* and *RAN*,¹⁶⁵ are involved in HCC risk. Some polymorphisms of *DNMT3a* and *DNMT3b* have been recently found in HCC,^{216,217} but they are not correlated with liver cancer risk. A *GNMT* polymorphism has been described and considered an early event in hepatocarcinogenesis,²¹⁸ but its association with susceptibility to HCC has not been unequivocally demonstrated.

Several observations indicate that the epigenetic manipulation of transcription and translation mechanisms is strongly involved in hepatocarcinogenesis (Figure 3).

Transcriptional deregulation implicates down-regulation of oncosuppressors and of *MAT1A*, leading to global DNA hypomethylation that contributes to signaling deregulation through the increment of GI and possible up-regulation of oncogenes with CpGs-rich promoters. Translational deregulation occurs via the action of RBPs on MATs, as well as by miRNAs affecting *MAT1A* expression or exhibiting oncosuppressor or oncogene activities. Finally, ubiquitination and proteasomal degradation of oncosuppressors represents an important posttranslational deregulation mechanism strongly involved in hepatocarcinogenesis.

These deregulation mechanisms are correlated with HCC prognosis, and further study on these correlations could allow discovering of new prognostic markers. Furthermore, epigenetic silencing of oncosuppressor genes in HCC, mediated by methylation of CpG islands in gene promoters, histone deacetylation or methylation, and silencing of oncosuppressor miRNAs by epigenetic alterations, offer the possibility of new epigenetic approaches to HCC treatment. Restoring the expression of tumor suppressor miRNAs may be a novel promising therapeutic strategy for HCC.

Acknowledgments

Supported by grants from Associazione Italiana Ricerche sul Cancro (IG8952) and Fondazione Banco di Sardegna.

Disclosure

The authors report no conflicts of interest in this work.

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