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Linking metabolism and epigenetic regulation in development of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common form of cancer globally and is rarely curable once detected. The 5 year survival rate of patients diagnosed with late stage HCC may be as low as 27 %. HCC is a cancer largely driven by epigenetic changes which arise from exposure to exogenous environmental factors, rather than coding sequence mutations. The liver is susceptible to effects from; Hepatitis C and Hepatitis B viruses, exposure to aflatoxin and continuous excessive consumption of alcohol. The liver is a highly metabolic organ balancing many vital biochemical processes; exposure to any of the above environmental factors is associated with loss of liver function and is a major risk factor for the development of HCC. Emerging studies aim to examine the underlying metabolic processes which are abrogated in cancer and lead to the altered flux and availability of key metabolites important for epigenetic processes. Metabolites have been shown to act as substrates for many canonical epigenetic regulators. These enzymes are responsible for regulating histone modification, DNA methylation and micro RNA expression. By studying the impact of altered liver metabolism we may better understand the long term epigenetic processes which lead to the development and progression of HCC.

Introduction

Hepatocellular carcinoma (HCC) is a major cause of cancer related deaths globally. The incidence of HCC is rising in westernized countries, and has been rising over the past few decades.[1, 2] The 5 year survival rate for HCC patients is low, and as of 2012 was estimated to be 27% (with the caveat of intrahepatic biliary cancer).[2] This high mortality is due to detection of late stage HCC and the recurrence of tumors following resection.[1] Hence, there is an urgent need to develop rapid and accurate diagnostic tools for early detection and treatment. The causes of HCC are heterogeneous, with both genetic and epigenetic alterations associated with tumor progression.[3–10] Infection with hepatitis C (HCV) and

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hepatitis B (HBV) are major risk factors, as are exposure to aflatoxin (found in stored peanut products) and chronic alcohol abuse.[2] The liver produces metabolites essential for epigenetic processes. De-regulation of both the hepatic one carbon and oxidative phosphorylation (TCA) cycles in the liver have major roles in driving aberrant epigenetic changes during tumor progression.[11–14] In a progressive model of HCC we may assume that infection of HCV, HBV or chronic alcohol abuse are initiators that cause functional changes in the liver which lead to HCC. Changes in both the TCA and hepatic one carbon cycles may provide good models to determine HCC progression.

Over 80 years ago Otto Warburg first reported that cancer cells preferentially convert glucose to lactate, even in the presence of sufficient oxygen for aerobic metabolism.[13] Subsequent studies have identified links between tumor cell function and the switching of ATP production from oxidative phosphorylation to anaerobic glycolysis.[15–17] Utilizing glycolysis as the primary source of energy production has several advantages for tumor cells. For example, by not producing mitochondrial reactive oxygen species (ROS), tumor cells have diminished capacity to undergo apoptosis.[17, 18] Also, increasing glucose uptake allows tumor cells to grow rapidly and out-compete healthy tissue for available glucose.[19, 20] Tumor cell metabolism can be linked to epigenetic changes during tumor progression, recent research has shifted focus from pure epigenetic studies to studies linking epigenetics with metabolic pathways. [18, 21, 22]

The purpose of this review is to summarize the current literature with particular focus on the epigenetic study of HCC. We also highlight here, some of the emerging-novel investigations that combine epigenetics with cancer metabolism. By identifying metabolic deficiencies in HCC we may develop new models by which we can study HCC.

Hepatocellular carcinoma as a CIMP+ cancer

So far, tissue testing has revealed no candidate genetic markers that can be associated with the majority of HCC cases. The frequency of TP53 mutation in HCC is highly variable between studies ranging from 0 to 63%, depending on the cohort analyzed.[3] Hepatocellular adenomas, from which HCC can arise, have been linked to β-catenin mutations.[23] In approximately 20–40% of cases β-catenin coding sequence mutations are associated with HCC.[24] With no common mutations of coding genes that can account for all cases of HCC it is likely epigenetic changes are the main driving mechanisms of HCC development. Mutations of coding genes which regulate epigenomic modifications may initiate HCC progression via changes to the epigenome, however these mutations are rarely observed.[17] During the course of HCC development alterations to global DNA methylation have been observed. For example, global hypomethylation leads to aberrant over-expression of oncogenes and genome wide chromosomal instability, which increases the risk of chromosomal translocations. [25, 26] Alongside global DNA hypomethylation, localized gene promoter hypermethylation frequently occurs.[25, 26] These two phenomena seem to be at odds and further study is required to elucidate the mechanisms driving these distinct processes.

HCC is characterised as a CpG Island Methylator Phenotype positive (CIMP+) cancer.[7,27, 28] CIMP+ is a term used to describe cancers exhibiting DNA hypermethylation at CpG Islands, where the number of promoters that become hypermethylated may increase throughout tumor development.[27, 28] Studies of gene promoter methylation have identified increasing DNA methylation in liver tissues from patients with HCC. The number of methylated gene promoters increases between cirrhotic and primary HCC tumor samples and between primary HCC and metastatic tumor samples.[7, 27] This is important as it implies that worsening DNA hypermethylation can drive tumor progression.

CIMP markers have been shown to have predictive value in the detection of HCC recurrence in HBV-associated HCC following liver transplantation.[28] Wu and colleagues (2010) studied the methylation profiles of 7 genes in DNA collected from tumor tissue from HCC patients and from normal healthy liver tissue. Patients were identified as having CIMP+ HCC by gain of methylation in a subset of genes. These patients had significantly lower recurrence free survival.[28] However, it is to be noted that HBV associated HCC is more readily studied by DNA methylation, as the hepatitis B virus protein X (HBx) indirectly increases expression of the *de novo* methyltransferase DNMT3a.[29]

Much research has been undertaken to identify hypermethylated CpG Island (CGI) promoters. CGIs are usually unmethylated and the presence of CpG methylation at gene promoters is associated with gene silencing. Cumulative promoter hypermethylation is an indicator of tumor stage, as more genes involved in tumor suppression or growth regulation become silenced over time. Epigenetic biomarkers based on promoter hypermethylation have been developed as diagnostic tools to detect liver cirrhosis and HCC (Table 1).[4–10] Many studies have shown that progressive promoter methylation accumulates throughout HCC development and is associated with tumor recurrence and prognosis.[27, 28, 30–32]

Genes often found to be hypermethylated in HCC, include those involved in gene expression (transcription factors), growth regulation, cell cycle progression and apoptosis (Table 1). [4–10] Many of the DNA methylation biomarkers identified distinguish between normal healthy tissue and cirrhotic tissue, however the distinction between cirrhotic tissue and HCC is less clear. Many gene promoters which are methylated in HCC are also methylated in the surrounding cirrhotic tissue (Table 1).[4–10] The incidence of gene promoter methylation for any given gene is also highly variable and differs from study to study, many of these markers are not essential for HCC and are only generally indicative of the mechanisms regulating tumor cell progression.

Given the paucity of genetic mutations and the length of time liver cancer takes to develop, one likely route by which HCC may develop is by abrogation of metabolic pathways. Reduced turnover of metabolites or changes to metabolite synthesis may influence epigenetic modifications overtime. One of the primary examples of these epigenetic changes has been studied in conjunction with the activity of the histone deacetylase SIRT1. SIRT1 represses glycolysis in the liver by interacting with and deacetylating histones at the promoters of several genes including FOXO1, CRTC2 and STAT3, in an NAD-dependent manner.[33] During glycolysis, energy overproduction reduces the availability of NAD+ which is reduced to NADH, as cancer progresses a lack of NAD+ reduces the activity of

SIRT1.[33] Studies of SIRT1 and NAD+ indicate that metabolite availability influences epigenetic modifications such as histone deacetylation enabling the expression of FOXO1, CRTC2 and STAT3. The paucity of NAD+ directly down regulates the activity of SIRT1 and is one mechanism by which the lack of a specific metabolite may affect the expression of a number of genes by directing epigenetic changes.

Epithelial to Mesenchymal transition, metastasis and microRNAs

Epithelial to mesenchymal transition (EMT) is an attractive model to study HCC as it involves the analysis of functional changes to gene expression which occur in epithelial tumor cells (ETCs). Although relatively confined, as ETCs transform into mesenchymal cells they become motile and invasive, and may enter the circulation.[34–36] Our laboratory has developed a novel cell line LH86 derived from a well differentiated model of HCC without the associated hepatitis or cirrhosis.[37] This LH86 cell line has been used in a novel mouse xenograft model of HCC/EMT to show that HGF and TGF-β-1 drive EMT via COX2, PGE2 and AKT gene overexpression.[36] However, there remains much work to be done on the characterisation of EMT. Another prominent area of research is metastasis which is also a primary cause of death. Even after successful surgical removal of primary tumors, metastatic cancer recurrence does not reduce mortality with less than 30–40% of patients surviving 5 years post resection.[38] This emphasizes the importance of studying changes in tumor cells before they enter the circulation (becoming circulating tumor cells, CTCs), undergo metastasis and spread to other parts of the body. EMT is required prior to tumor cell invasion and is characterised by key molecular changes including loss of E cadherin, gain of expression of Vimentin, collagen I and fibronectin. There is also an associated increase in the expression of several transcription factors including Twist, SLUG, SNAIL family members and Zinc finger E box containing transcription factors, ZEB1 and ZEB2.[36, 40, 41] Furthermore, EMT has also been shown to be characterized by aberrant microRNA (miRNAs/miRs) expression. miRs are small highly conserved RNA molecules which regulate gene expression by binding to the 3′-untranslated regions of target mRNAs.[42–44] Consequently, miRs silence gene expression and can also act on multiple gene targets.[45, 46] Over 20 miRs have been shown to be aberrantly expressed in HCC and EMT phenotypes, miRs have also been studied to identify markers of HCC and other cancers, and may be overexpressed or down regulated causing aberrant expression of tumor suppressors or oncogenes.[42–50] Both miR-200c and miR-141 expression are lost in many cancer types, DNA methylation of a putative CpG island upstream of the 200c/141 cluster has been shown to repress the expression of these miRs.[42,43] By directly targeting ZEB1 and ZEB2 miR-200c and mIR-141 alter the expression of E-cadherin and other genes involved in cell polarity. miR-200c has been shown to regulate EMT, in cancer the loss of miR-200c due to gain of DNA methylation also prevents repression of the neurotropic receptor tyrosine kinase 2 (NTRK2). [51] NTRK2 expression confers anoikis resistance and enables cells to survive after they detach from the extracellular matrix and invade the circulation.[51] miR-200c restoration could be used as a therapeutic intervention to induce anoikis in circulating tumor cells preventing the spread of cancer.

Several studies also provide evidence to support the hypothesis that aberrant miRNA expression may confer chemoresistance in certain cancer types. DNA methylation of

miR-193-3p confers 5-fluorouracil (5-FU) resistance in HCC. In normal healthy cells SRSF2 expression regulates the pro-apoptotic splicing form of Caspase 2 (CASPL). The loss of DNA methylation in HCC allows the expression of miR-193-3p which in turn suppresses SRSF2 and subsequent up regulation of CASP2L, making the cells resistant to apoptosis induced by 5-FU. [50] The de-regulated expression of miRs is a feature common to many cancer types, and the study of microRNA expression provides another route whereby diagnostic and prognostic tools could be developed to understand cancer stage and risk. One caveat of miRs however, is that due to their small length (~22–24 nucleotides) they may target hundreds of coding genes making their direct effects difficult to study.

The mechanisms causing aberrant gene expression of genes driving EMT have not been fully elucidated. However, by incorporating studies of EMT with metabolomics and epigenetics, models for changes in gene expression could be developed to more fully understand the processes involved.

The continuing study of miRs may yet elucidate powerful predictors of tumor stage and aggression; and provide targets to induce tumor-sensitivity to chemotherapy. These markers may also be combined with associated DNA methylation markers for further diagnostic accuracy or as a putative target for miRNA restoration. A recent study of HCC caused by HCV identified a number of mIRs with altered expression which may indicate that different subsets of HCC have different miR profiles. [81]

Evidence from mouse studies has shown that the expression of miRs in primary hepatocytes may be affected by the availability of certain adenine analogs.[52] 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) is an analogue of adenine and treatment of murine models of HCC, with exogenous AICAR has been shown to alter the expression of 41 miRs in primary hepatocytes indicating a relationship between miR expression and AMP kinase (AMPK) mediated energy sensing. However, the mechanism by which miR expression modulates AMPK remains to be determined. [52]

Role of the hepatic one-carbon cycle in epigenetic regulation

Another mechanism whereby metabolic and epigenetic processes intersect is the methionine synthesis pathway (or hepatic one-carbon cycle) and its components, which are central to liver function. More than half of the dietary methionine is converted into S-Adenosyl methionine (SAM) where over 85% of all methylation reactions take place.[53] This makes the liver a unique driver of epigenetic processes with the labile methyl pool and the ratio of SAM and adenosyl-homocysteine (AdoHCY) regulating essential to liver function. SAM as a methyl donor has a diverse range of functions and is used a substrate for reactions including: methylation of DNA, histones, phospholipids and small molecules, as well as the synthesis of polyamines.[54] Key enzymes involved in the hepatic one-carbon cycle include; methionine adenosyl transferase (MAT), S-adenosyl hydrolase (SAH), betaine homocysteine methyltransferase (BHMT), Glycine-N methyltransferase (GNMT) and cystathione β synthase (CBS) (Figure 1).

Many studies utilizing cell culture and mouse knockout models have helped to clarify the function of many one-carbon cycle genes, with the abrogation or knockout of these genes

leading to liver disease and HCC.[55–57] Avila *et al* identified that a number of key one-carbon cycle enzymes are under-expressed in HCC.[58] In cirrhotic samples a reduced abundance of mRNA was identified in the following genes; GNMT, methionine synthase (MS), BHMT and CBS. Loss of expression was also identified in BHMT, CBS, GNMT and MS in tissues taken after resection from HCC patients and compared to normal liver tissue.[58] These data indicate that abrogation of the TCA cycle may be a predisposing factor to developing HCC in a cirrhotic background.

Methionine adenosyltransferase (MAT1A) converts methionine into SAM and is also expressed exclusively in the liver. MAT1A is epigenetically regulated with studies of MAT1A identifying 2 CpG sites in the first exon, the methylation of which, correlates with reduced MAT1A expression.[17, 58] De-regulation of MAT1A is associated with HCC development and is in turn linked to the overexpression of hepatocyte growth factor HGF. The primary function of HGF is to expand the liver during development and to drive regenerative growth following injury or insult. HGF is present in the circulation with concentrations increasing after partial hepatectomy, liver injury or fulminant hepatic failure.[59] Chronic hepatic injury, as caused by hepatitis and cirrhosis, allows HGF to be constitutively expressed, this in turn drives hepatocyte expansion, placing a strong selective pressure towards tumorigenesis and the development of HCC.[54] Interestingly, hepatic HGF overexpression correlates well with the switch in expression of MAT1A to MAT2A in HCC.[54] HGF also drives growth of cells at low density but inhibits growth at high cell densities. However, the precise mechanism by which HGF and MAT2A interact has not been fully described.[60, 51]

Studies of the hepatic one-carbon cycle provide evidence for the global hypomethylation component of HCC. Down-regulation of one or more of the cycle's constituent enzymes may reduce the availability of the hepatic labile methyl pool, but further studies are necessary. De-regulation of the hepatic one-carbon cycle has not been linked with promoter hypermethylation; therefore this process must be controlled by other mechanisms during HCC development.

Warburg effect on epigenetic processes

Under the partially hypoxic conditions found in many types of solid tumor, cells switch to glycolysis as the primary source of energy production.[62] This change in metabolism which may also occur in normoxia has been linked to stabilization of HIF1α. The HIF1 heterodimer is a transcription factor which controls a suite of genes involved in the up regulation of glycolysis. The switch that takes place in non-hypoxic conditions is linked to rare mutations which alter the Hif Prolyl hydroxylase (PHD or EgIN) proteins; these in turn modify HIF1α for ubiquitination by Von-Hippel Lindau protein (pVHL). This change may also be linked to mutations of the pVHL protein which recognizes hydroxylated HIF1α and marks it for ubiquitination.[17, 63] Either of these mechanisms permit the stabilization of HIF1α in the cytoplasm allowing it to form a heterodimer with HIF1β. This complex can then translocate to the nucleus where it acts as a transcription factor up-regulating the down-regulate the TCA cycle.[14, 17,20,63, 64] One of the major consequences of depressing the TCA cycle is the reduced availability of α-ketoglutarate. This decreases the activity of α-

ketoglutarate dependent proteins which are responsible for hydroxylating many substrates in the cell important in epigenomic control. Of primary interest are the Ten Eleven Translocation (TET) family of FeII-dependent, α-ketoglutarate-dependent methyl dioxygenases which convert methylated cytosine to 5 hydroxy methylcytosine (5hmC).

We hypothesize that loss of TET activity may explain gene promoter hypermethylation in HCC and other CIMP+ cancers. TET proteins confer the epigenetic modification of a hydroxyl group to 5 methylcytosine (5mC) to form 5hmC. 5hmC is a demethylation modification and its loss has been implicated in cancer development.[65–67] First identified in 1953 in the bacteriophage T2.I, 5hmC functions in the bacteriophage genome in defence against excision by host enzymes.[68] Recently this modification was identified in murine brain and ES cells, Purkinje neurons and in human embryonic kidney cells with induced overexpression of TET1.[65, 66] The discovery of the TET family and its role in conferring the epigenetic mark 5hmC represents a major breakthrough in epigenetics and cancer research. Previously the regulation of DNA methylation remained enigmatic as no de-methylation enzymes had been identified that could account for the genome wide modification of DNA methylation. These discoveries have significant implications for previous investigations using bisulfite conversion to study DNA methylation since bisulfite sequencing and the use of methylation sensitive restriction enzymes, are unable to distinguish 5mC from 5hmC. There is, however, a new technique which is able to differentiate 5hmC from 5mC.[69]

TET proteins have been suggested to modify genomic DNA methylation in two ways; either by binding to unmethylated CpGs via a CxxC binding domain thereby preventing DNA methyltransferase activity, or by hydroxylation of 5mC marking it for subsequent demethylation.[70] TET2 does not contain a CxxC binding domain but does possess a role in cancer development and may have multiple binding partners allowing it to modify alternative sites in the genome.[70] Several mechanisms are postulated whereby methylated DNA can be demethylated. One is base excision repair (BER), while the other is modification by TET members followed by BER, although the addition of the hydroxyl group to 5mC alone may be sufficient for passive demethylation.[71] Genomic distribution of 5hmC was first studied using an immunoprecipitation technique, 5hmC-IP-seq, which combines an antibody-specific for the enrichment of 5 hydroxyl-methylcytidine, and subsequent massive parallel sequencing of 5hmC-enriched DNA.[72] Since these important initial studies, investigations have detected 5hmC in human tissues.[72, 73] Loss of TET expression (which confers the 5hmC modification) leads to global genomic reduction of 5hmC and has been detected in several cancers including HCC.[74, 75] 5hmC also has a protective role in gene bodies, enabling transcription by preventing localization of methyl binding proteins,[61, 62] thereby preventing transcriptional silencing associated with DNA methylation.

Thus far, detection of 5hmC in cancer has been performed by histological staining and other global methods which measure total 5hmC. 5hmC genomic distribution has been studied in human stem cells and mouse studies of 5hMC and TET distribution have also been conducted.[76, 77]

Future Directions

Since the observations made by Warburg almost 90 years ago,[13] significant strides have been made to understand the molecular mechanisms whereby altered tumor cell metabolism may drive tumor progression. The field of cancer epigenetics has sought to identify how gene expression is modified during tumor development and studies of DNA methylation have offered insights into the genome wide epigenetic processes which occur. Many studies have sought to identify DNA methylation biomarkers of HCC, however there are few that could be applied clinically as methylation of specific gene targets is highly variable (Table 1). Better understanding of the methionine synthesis pathway, may be able to more accurately determine which genes are likely to become hypomethylated and drive HCC development.

Another epigenetic mechanism that may yield promising results is the study of microRNAs. miRs are often aberrantly expressed in HCC and have been shown to play a role in EMT. Future studies may seek to identify specific miRs required for HCC development and the subset of genes targeted by mIRs for post translational regulation. Further metabolic studies linking miR expression with nucleic acid metabolism may provide insights into restorative mechanisms of miR expression.

Studying the enzymes down-regulated in glycolytic tumor metabolism could also be vital in understanding cancer. The TET family genome distribution could unveil the mystery of gene promoter hypermethylation in CIMP+ cancers. Currently, we do not yet understand whether individual TET members target different genes exclusively or if they have a synergistic function (the answer may be both, and dependent on the presence of binding sites). Loss of TET expression could result in the aberrant hypermethylation observed in CIMP+ cancers, as TET may function to block the action of DNA methyltransferases at gene promoters. Another likely scenario is that TET expression dynamically regulates methylation marks which are laid down during cell division and may otherwise accumulate. Future studies may ascribe a role for TET expression and regulation of DNA methylation during EMT. By following methylation and hydroxymethylation during EMT we may be able to more fully understand how aberrant TET expression could drive tumor cell invasion and the development of circulating tumor cells.

Seeking therapeutic agents to restore oxidative phosphorylation could be a viable treatment option. By reinstating components of the TCA cycle such as α-ketoglutarate, the function of epigenetic modifiers such as EgINs, LOX and histone demethylases may also be restored.[17] In this regard, studies undertaken to assess the effect of Dichloroacetate (DCA) showed positive results *in vitro*.[78] However, due to the impermeability of DCA to cells the dosages required to treat HCC caused peripheral neuropathy in rats.[79] Conversely, the cell-permeable α-ketoglutarate showed promise in reversing competitive inhibition of EgIN proteins by fumarate and succinate in cancer cells, illustrating the potential for the development of pharmaceuticals that could restore TCA cycle function.[80] More *in vivo* experiments are required to test the efficacy of TCA metabolites in murine xenograft models of HCC, to identify the putative benefits in the treatment of HCC.

Comparative *in vitro* studies of HCC cell lines with different TET expression could clarify the epigenetic *vivo* studies of HCC cell lines in mice may also identify to what extent the loss of TET proteins play a role in cancer tumorigenicity and their response to anti-cancer chemotherapy. By studying the distribution of TET proteins in both hepatocyte and tumor genomes it may be possible to readily identify the genes effected by loss of TET expression and to use the data as candidate markers for tumor development, malignancy and recurrence. By approaching HCC in this way we may develop models for the study of all CIMP+ gastrointestinal cancers.

With the advent of genome wide platforms we are now able to study the multivariate changes occurring during cancer. By understanding how metabolic changes drive epigenetic changes during HCC progression, we can study the impact of altered metabolite availability. New investigations combining both tumor metabolism and epigenomics will be pivotal in developing future models by which we can identify the causes of HCC and other CIMP+ cancers.

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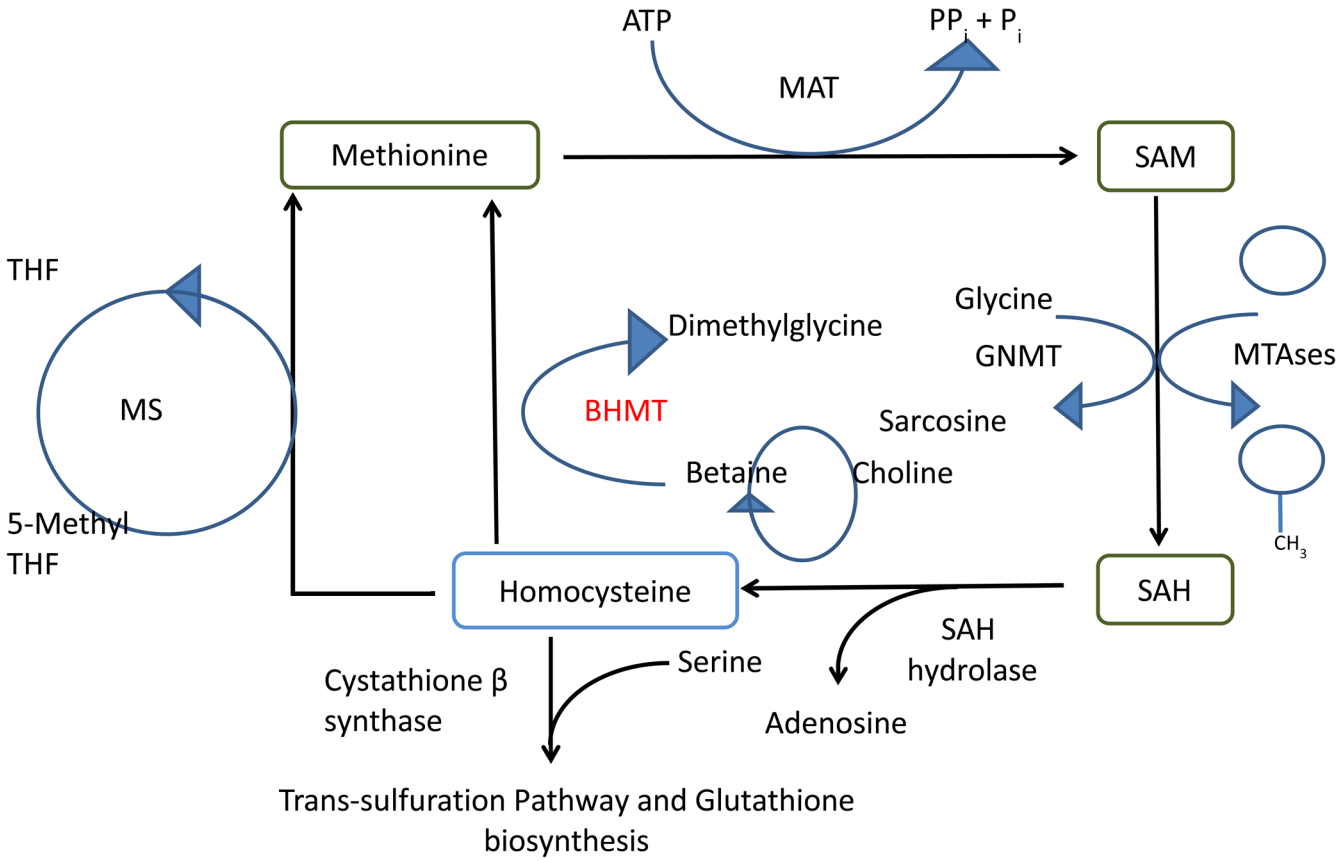

Figure 1. Schematic showing the hepatic carbon-one cycle

SAM biosynthesis is catalyzed by methionine adenosyltransferase MAT1A in the liver. SAM donates methyl groups to methyltransferases and also Glycine N-methyltransferase for methylation of Glycine to become S-adenosyl homocysteine, which is hydrolyzed to form Hcy. Hcy is then re-methylated to form methionine by one of two enzymes, methionine synthase (MS), the canonical enzyme found in all cells, or betaine-homocysteine-methyltransferase BHMT which is found mainly in the liver and kidney. Low abundance of SAM can lead to a lack of methyl reactions in the liver, oxidative stress (via deregulation of the transulfuration pathway) and arrest of DNA synthesis (via the folate metabolic pathway). Maintenance of the SAM: Hcy ratio and the labile methyl pool are essential to liver function.

Table 1

Aberrant DNA methylation markers for hepatocellular carcinoma

Synonyms of gene promoters are provided and presented alongside their genomic location. The frequency at which gene promoters are found to be hypermethylated are given for HCC tissues (HCC), cirrhotic tissues (CIR) and adjacent normal tissues (N).

Gene	Location	Function	Methylation frequency %			Refs
			HCC	CIR	N	
APC	5q22.2	Prostaglandin Synthesis	53–81	13–44	0–14	7–9, 12
BLU	3q21.3	Unknown Zinc Finger	20	-	-	11
COX2	1q31.1	Prostaglandin Synthesis	35–50	0	0	8, 9, 12
CPS1	2q34	Urea cycling	80	0	0	13
DAPK1	9q21.33	Apoptosis	10	10	0	8
E2F-1	20q11.22	Transcription factor	70	24	0	10
E-Cadherin	16q22.1	Cell Adhesion	33–67	6–20	7	7–9
GSTP1	11q13	Glutathione Synthesis	41–76	13–31	0–7	7–9
hMLH1	3p21.3	Mismatch repair	0	0	0	8
P14	11q13.1	CDK Inhibitor	6	0	0	7
P15	18q12.2	CDK Inhibitor	42–47	0–19	0	7,10
$P16^{INK4a}$	9q21.3	CDK Inhibitor	16–83	0–23	0–10	7–10, 12
P21	6p21.2	CDK Inhibitor	63	53	10	10
P27	12p13.1	CDK Inhibitor	48	36	0	10
P300	22q13.2	Growth/Cell division	65	54	0	10
P53	17p13.1	Tumor suppressor	14	8	0	10
P73	1p36.32	Tumor suppressor	6	0	0	7
RaR-Beta	3p24.2	Retinoic acid signalling	12	13	7	7
RASSF1A	3p21.3	Apoptosis	59–75	0–55	0	8,9,11,12
RB	13q14.2	Chromatin Structure	32	25	0	10
SEMA3B	3p21.3	Apoptosis	83	-	-	11
SOCS-1	16p13.13	Cytokine inhibitor	43–65	28–7	0–7	7,12
TIMP3	22q12.3	Cell adhesion	13	0	0	8
WT1	11p13	Urogenital Devpt	54	18	0	10