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Epigenetic mechanisms involved in the pathogenesis of hepatobiliary malignancies

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Abstract

Primary tumors of the liver and biliary tree are increasing in frequency and portend a miserable prognosis. Epigenetic regulation of gene expression has emerged as a fundamental aspect of cancer development and progression. The molecular mechanisms of carcinogenesis in hepatocellular carcinoma and cholangiocarcinoma involve a complex interplay of both genetic and epigenetic factors. Recent studies investigating the possible epigenetic mechanisms induced in the disease have shed new light on the molecular underpinnings of hepatobiliary cancers. In addition, epigenetic modifications of DNA in cancer and precancerous lesions offer hope and the promise of novel biomarkers for early cancer detection, prediction, prognosis and response to treatment. Furthermore, the reversal of epigenetic changes represents a potential target for novel therapeutic strategies and medication design.

Keywords

5-azacytidine; cholangiocarcinoma; epigenetics; HCC; hepatocellular carcinoma; histone modification; hypermethylation; hypomethylation; methylation; p16; RAS; sorafenib; zebularine

Cancers arising from the liver parenchyma and biliary tree are both common and deadly. Hepatocellular carcinoma (HCC) typically occurs in the setting of chronic liver disease, particularly cirrhosis. Numerous etiologies exist, such as viruses hepatitis B and C, and heavy alcohol use. HCC is the third leading cause of cancer death in the world [1], and more than 500,000 cases are diagnosed annually [2]. Cholangiocarcinoma (CC) usually develops in the setting of chronic biliary injury, that is, primary sclerosing cholangitis, and is also increasing in incidence [3]. CC is subdivided into intra- and extrahepatic lesions, and these subtypes differ with respect to etiology, treatment and prognosis. Overall, effective treatments for CC are lacking, and, accordingly, this diagnosis portends a miserable prognosis.

Epigenetics is defined as the heritable changes in gene expression that are, unlike mutations, not attributable to alterations in the sequence of DNA. Two predominant epigenetic mechanisms are DNA methylation and histone modification. Epigenetic regulation of gene

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expression has emerged as a fundamental pathway in the pathogenesis of numerous malignancies [4]. Cancers of the digestive system are no exception. In fact, many exciting discoveries regarding epigenetics in general have been made by studying cancers of the liver and hepatobiliary tree. It is now accepted that there is a complex interplay of genetic and epigenetic abnormalities that accumulate in precancerous tissues and culminate in the development of full-blown carcinoma [4–6]. Epigenetic modifications of DNA in cancer and precancerous lesions offer hope and the promise of novel biomarkers for early cancer detection, prediction, prognosis and response to treatment. Furthermore, reversal of epigenetic changes represents a potential target of novel therapeutic strategies and medication design [7]. There has been an explosion of data and studies published in the last 5 years regarding the epigenetic mechanisms involved in the pathogenesis of HCC and CC. This review aims to provide a critical summary of the most salient and provocative studies related to this topic.

DNA methylation

DNA methylation is the addition or subtraction of a methyl group to a cytosine nucleotide in a DNA sequence. Methylation is controlled by a family of specific enzymes known as DNA methyltransferases. The addition of methyl groups, or hypermethylation, can be highly specific to a particular gene. Regions of the genome rich in sequences of a cytosine preceding a guanine (CpG dinucleotides) are known as CpG islands. In fact, CpG islands exist in the promoter regions of approximately half of all genes. The hypermethylation of CpG islands in the promoter region of a gene can result in transcriptional silencing of the gene and subsequent loss of protein expression. Thus, in addition to allelic loss and mutation, hypermethylation of tumor suppressor genes is now recognized as an alternative means of gene silencing important in the development of cancer [8]. The hypermethylation of genes involved in the cell cycle, DNA repair, angiogenesis, metabolism of carcinogens, apoptosis and cell–cell interaction have been implicated in carcinogenesis. Methylation can also inhibit the transcription of miRNA, resulting in tumorigenesis. It should be noted that epigenetic phenomena also occur as part of normal physiological processes, such as the natural course of aging, when certain genes become hypermethylated [4]. In fact, hypermethylation of repetitive sequences in the genome are theorized to prevent chromosomal instability [4,9]. DNA hypermethylation is also required for genomic imprinting, a process that results in the monoallelic expression of genes [10], and is involved in the inactivation of the second X chromosome (Barr body) [11].

Rather than gene-specific hypermethylation, a genome-wide decrease in DNA methylation was one of the first epigenetic alterations to be linked with cancer development [12]. Indeed, a poignant feature of all tumors is a global reduction in methylation [13]. Although CpG islands are frequently found in the regulatory regions of genes, the majority of CpG dinucleotides are actually found in the intergenic portions of the genome [14]. Hypomethylation is most frequently observed in repeated sequences of DNA within these introns, and is associated with genomic instability and tumor progression [14,15]. These repetitive sequences may be transposable elements (transposons) or DNA satellites that are normally methylated and found throughout the genome [7]. During the neoplastic process, genomic hypomethylation increases as a tumor progresses from a proliferative nodule to an invasive carcinoma [4,16].

Why aberrant methylation occurs is not fully understood [4,8]. It is recognized that certain genes are methylated in an age-related fashion [17], while others are methylated in a cancer-specific pattern [4,8]. Indeed, one carcinogenetic pathway of particular relevance to HCC is the CpG island methylator phenotype (CIMP). CIMP-positive cancers have distinct clinical, pathologic and genetic features (see the paragraphs that follow). Whether carcinogens, diet (e.g., folate) or other environmental factors contribute to methylation remains to be elucidated and is an area of active research.

In the laboratory, DNA methylation can be measured by many different methods in tissues, and occasionally in peripheral blood or other body secretions, such as bile. One of the major advantages of attempting to detect methylation is the inherent stability of DNA. Before the advent of DNA methylation sequencing methods, isoschizomers with different methylation sensitivities were used to detect DNA methylation. A major disadvantage of this method is that less than 5% of the methylated cytosine residues can be assessed in any given DNA sequence. Since the early 1990s, a key method by which methylation levels are determined requires an initial bisulfite conversion of DNA. This bisulfite treatment converts unmethylated, but not methylated, cytosines to uracils. Subsequent gene-specific methylation can be determined by qualitative or quantitative methylation-specific PCR (MSP) using primers and probes specific to the corresponding methylated DNA sequence [18]. The advantages of MSP is that it gives a positive display of methylated cytosines and provides the entire profile of methylation for a defined DNA sequence, rather than the assessment of just a few cytosines within a sequence. Real-time quantitative MSP is preferred by the author, as it determines the actual percentage of methylated alleles in a given sample.

DNA sequencing can also be performed on bisulfite-converted DNA in order to determine specific regions of hyper- or hypomethylation. This is a particularly useful technique to determine regions of differential methylation, and aids in primer and probe design for more specific MSP. Pyrosequencing is a method of real-time DNA sequencing that is based upon the activity of DNA polymerase and relies on the luminometric detection of pyrophosphate release after nucleotide incorporation [19]. An advantage of pyrosequencing is that it combines the high-throughput nature of PCR-based technologies with the ability to analyze all of the individual CpGs of a given region [19].

A limitation of the PCR-based technologies is that they specifically target candidate genes of interest. More recently, high-throughput, genome-wide, microarray platforms have been developed in attempt to define the global methylation pattern of tumors. Methylated DNA immunoprecipitation is an immunologic approach that enriches methylated DNA, and is based upon the principle that genomic DNA is randomly sheared by sonication and can be immunoprecipitated with an antibody that specifically targets 5-methylcytidine. This technique can be used to generate comprehensive, genomic DNA methylation profiles and to identify abnormally (hyper- or hypo-) methylated genes in cancer cells [20].

The methylated CpG island amplification method is based on the digestion of genomic DNA with the methylation-sensitive restriction enzyme, *Sma*I, which cuts only at unmethylated sites, leaving blunt ends between the cytosine and guanine. The DNA is then digested with the methylation-insensitive *Sma*I isoschizomer, *Xma*I, which leaves a four-base overhang. These two serial digests are followed by ligation of adaptors to the overhang, and finally, performing adaptor-specific PCR amplification [21]. Thus, this method results in the enrichment and amplification of methylated DNA fragments only. These methylated DNA fragments are then used for interrogations with microarrays platforms.

An additional method by which to discover novel targets of methylation-induced transcriptional silencing is to treat cancerous cell lines with agents that reverse epigenetic events, and then perform gene-expression microarrays to determine which genes become upregulated.

Histone modification

Histones are the protein components of chromatin, the structure around which DNA is wound. Histones also participate in the regulation of gene expression. There are several types of post-translational modifications that can affect histones, including methylation, acetylation, phosphorylation and ubiquitination. These modifications can affect interactions between DNA

and histones, leading to alterations in gene transcription, DNA repair, DNA replication and even the organization of chromosomes [4]. The full spectrum of histone modifications and all their combinations form a remarkably complex network of genetic regulation. Modifications can occur in different histone proteins, residues and variants. Alterations can involve different chemical structures, such as acetyl groups, methyl groups and phosphate ions. Histones can be mono-, di- or trimethylated. In general, histone acetylation is associated with transcriptional activation, and deacetylation is linked with transcriptional repression. Thus, deacetylation is implicated in the silencing of tumor suppressor genes in carcinogenesis. The effect of histone methylation depends on the amino acid affected, and the amino acid location in the histone tail [4,22]. Further complexity is observed when hypermethylation and histone modification work in concert to alter gene transcription. For example, some cancers exhibit CpG island hypermethylation in combination with multiple histone modifications, such as deacetylation of histones H3 and H4, methylation of histone H3K9, trimethylation of histone H3K27, and loss of trimethylation of histone H3K4 [4,23,24]. Finally, genetic lesions, such as altered expression of the enzymes that modify histones, can also contribute to the overall milieu of epigenetic abnormities witnessed in cancer cells [4]. Given the wide range of downstream detrimental effects that histone modifications can incur, they are a logical target of anticancer therapy. Indeed, histone deacetylase (HDAC) inhibitors are in early-phase clinical trials for the treatment of several cancers, with promising results [25].

In the laboratory, histone modifications are readily and accurately detectable by mass spectrometry. However, this technique is laborious and requires highly specialized training and equipment [26]. In order to determine the true biological significance of histone modification, DNA sequence information is also required. The optimal technique, chromatin immunoprecipitation, combines sequencing technology with DNA that has been immunoprecipitated with antibodies against specific histone modifications. Genome-wide studies of histone modifications are now possible through the use of so-called ‘chromatin immunoprecipitation on chip’ studies that couple immunoprecipitation with microarray sequencing platforms. The current limitations of this technique are the quality of the polyclonal antibodies engineered against the histone modifications [26].

Hepatocellular carcinoma

The etiopathogenesis of HCC is quite complex, and is yet to be fully elucidated. This complexity is likely to be, in large part, owing to the multitude of etiologies that can cause chronic liver disease and cirrhosis. The etiologies include hepatitis viruses (B, C and D), alcohol exposure, nonalcoholic fatty liver disease, iron overload and autoimmune liver disease. Additional factors that do not cause liver disease but increase the risk of HCC in a patient with liver disease include aflatoxin exposure in Subsaharan Africa and East Asia, tobacco, male sex, advanced age, obesity and diabetes mellitus. The ultimate risk factor for HCC is cirrhosis; however, with improved awareness and methods of detection, HCC is increasingly being diagnosed in patients with advanced fibrosis. The notable exceptions are aflatoxin exposure or chronic hepatitis B infection, which may cause HCC in the absence of significant liver fibrosis [27–30]. The generally accepted paradigm is that, as chronic hepatitis leads to fibrosis, genetic and epigenetic alterations gradually accumulate. Mutations, loss of heterozygosity, activation of oncogenes and the epigenetic silencing of tumor suppressor genes all play important roles in the development of cancer. Once the liver becomes cirrhotic, clonal selection can occur and preneoplastic lesions known as dysplastic nodules form [31]. These lesions then accumulate more genetic and epigenetic abnormalities, and can eventually degenerate into a well-differentiated HCC. Why some patients with cirrhosis develop HCC and yet the majority do not remains unknown. Pertinent to this review, it appears that the hypermethylation of certain genes occurs early in this neoplastic process, that is, in cirrhosis, and therefore these genes may represent candidates for early-detection biomarkers.

Hypomethylation

Global (i.e., genome-wide) decreases in methylation, or hypomethylation, are a major signature of cancer cells [32]. Hypomethylation is most functionally relevant when it occurs in coding portions of genes, leading to alternative transcripts or levels of mRNA. Global DNA hypomethylation is associated with activation of proto-oncogenes, such as c-JUN and c-MYC [33]. In addition, it is theorized that hypomethylation contributes to carcinogenesis by favoring mitotic recombination, leading to deletions, translocations and chromosomal rearrangements, collectively known as genomic instability. Overall, most DNA found in tumors is hypomethylated, with occasional gene-specific hypermethylation as described later [34]. In general, hypomethylation increases as the tumor progresses. Accordingly, hypomethylation of repetitive DNA elements such as SAT2, LINE1 and ALU occur in the multistep process of hepatocarcinogenesis, and correlate with a poor prognosis [35]. One important study examined the hypomethylation profiles of two groups of liver cancer patients that were segregated based upon their survival and gene-expression profiles [36]. When compared with noncancerous tissue, hypomethylation was significantly increased in the liver cancer tissue from both patient subclasses. Further analysis revealed a high degree of correlation between global hypomethylation and genomic instability in liver cancers [33]. Moreover, hypomethylation in liver cancer does not appear to be specific to any of the different etiologies of HCC, indicating that this epigenetic event is an important process in hepatocarcinogenesis [33].

Hypermethylation

As with most cancers, numerous derangements of normal cell biology have been described in HCC. Multiple signal transduction pathways are abnormal in theses tumors, including the WNT/β-catenin pathway, the p53 pathway, the retinoblastoma pathway, the MAP kinase pathway, the RAS pathway and the JAK/STAT pathway [37]. Hypermethylation and subsequent loss of gene expression and the cognizant proteins involved in these pathways can affect cell biology, predisposing the cell to uncontrolled proliferation, loss of apoptosis and dysregulated adhesion and migration, all of which are the hallmarks of cancer.

Cell proliferation

Several pathways adversely affected in HCC affect cell proliferation. For example, the canonical WNT/β-catenin pathway is often disturbed in cancers related to alcoholic liver disease and viral hepatitis (B and C). The epigenetic inactivation of important WNT regulators can precipitate aberrant WNT/β-catenin signaling, leading to abnormal cell proliferation and survival. Specifically, hypermethylation-induced inactivation of the tumor suppressor gene adenomatous polyposis coli (*APC*) occurs in up to 81% of HCC, and leads to accumulation of β-catenin in the cell nuclei, where it functions as a putative transcription factor with oncogenic properties [38]. Interestingly, *APC* methylation is also frequently detected in tissues surrounding tumors, suggesting that methylation of this gene is not specific for hepatocarcinogenesis [39].

The retinoblastoma (pRb) protein is a potent tumor suppressor, and the activities of multiple cyclin-dependent kinases (CDKs) are linked with pRb phosphorylation and cell cycle progression [40]. Several important CDK inhibitors, namely $p16^{INK4A}$, $p21^{(WAF1/CIP1)}$ and $p27^{Kip1}$, are independently downregulated in up to 90% of HCC cases [37]. Reduced or absent expression of these proteins results in the loss of cell-cycle checkpoints and dysregulated cell proliferation. Importantly, the two major mechanisms by which $p16^{INK4A}$ expression is lost in HCC are via hypermethylation and the loss of heterozygosity of chromosome 9p21. Indeed, multiple studies have detected $p16^{INK4A}$ methylation with frequencies as high as 85% [41–44]. $p16^{INK4A}$ promoter methylation has also been reported in preneoplastic dysplastic nodules [45] and in the serum of patients at the time of their diagnosis [46]. Furthermore, one study detected $p16^{INK4A}$ promoter methylation in the blood samples of 44% patients prior to their

diagnosis of HCC [47]. Methylation and loss of *p16*^{INK4A} expression is closely linked with *p27* inactivation, leading to increased cell proliferation and predicting a poor prognosis [48]. There is increasing evidence that the hepatitis B X protein can induce methylation of *p16*^{INK4A}, supporting the close relationship between HCC and viral liver disease [49]. Taken together, these findings indicate that *p16*^{INK4A} is implicated in the pathogenesis of HCC and may be useful as a biomarker of early cancer detection.

Another gene in the pRb pathway that is silenced by hypermethylation in approximately 50% of HCC is *p15* ^{INK4B} [44]. Methylation of this gene has also been detected in the sera of patients with HCC [47,50].

The RAS pathway and its associated proteins are GTP-binding proteins that serve as molecular triggers that influence cell growth and differentiation. The binding of growth factors, such as EGF and IGF-1, to their receptors stimulates RAS activation, which in turn induces c-Raf, ERK and MEK activation. ERK then becomes phosphorylated, which triggers transcription factors such as c-Jun to regulate the expression of genes involved in cell growth and proliferation [51]. Unlike colon cancer, mutations of RAS are uncommon in HCC [52]. However, a pair of tumor suppressor genes that work together to inhibit the effects of RAS, RASSF1A and NORE1A, are inactivated by hypermethylation in 80–90% and 44% of HCC, respectively [35,51]. Methylation of RASSF1A is also frequently detected in the serum of patients who subsequently develop HCC, suggesting that this gene may also be a potential diagnostic biomarker [47].

The JAK/STAT signaling pathway plays an important role in cell proliferation and differentiation. The ubiquitous activation of the JAK/STAT pathway has been reported in HCC [53]. One possible mechanism for this activation is the methylation and inactivation of suppressors of cytokine signaling (SOCS1 and 3). SOCS1 and 3 are negative regulators of the JAK/STAT pathway, and methylation of their promoter regions has been detected in 30–60% of HCC cases [54].

Apoptosis

The loss of apoptosis is a common event in cancer development, and HCC is no exception. The key regulator of apoptosis, *TP53*, is mutated in approximately 50% of HCC. The percentage of *TP53* mutation is related to the origin of the population studied as well as the etiology of liver disease. Hepatitis B and aflatoxin-induced HCC are most commonly associated with TP53 mutations [37]. However, there are other important mediators of apoptosis that are affected by epigenetic phenomena in HCC. Acting as an initiator of caspase, caspase 8 (CASP8) is a significant regulator of apoptosis through the mitochondrial and death receptor pathways. Hypermethylation and subsequent inactivation of *CASP8* has been detected in up to 72% of HCC cases [55]. Other pro-apoptotic genes that are silenced by promoter methylation in HCC include *RASSF1A*, death-associated protein (*DAPK*) and X-linked inhibitor of apoptosis factor-1 (*XAF1*) [56,57].

Cell adhesion & migration

The loss of normal cell–cell adhesion results in tumor progression, invasion and metastases. A critical gene that mediates cell adhesion is E-cadherin (*CDH1*). Expression of this gene is lost in most tumors of epithelial origin [54]. Hypermethyation of *CDH1* has been detected with increasing frequency in dysplastic nodules (7.7% of nodules), stage 1 HCC (38.5% of tumors) and stage 2 HCC (52.9% of tumors) [58]. These findings suggest that *CDH1* methylation is an important event in hepatocarcinogenesis. Other members of the cadherin family, H-cadherin (*CDH13*) and M-cadherin, are also methylated in HCC, with varying degrees of frequency [54].

The expression of tissue inhibitor of metallo-proteinase-3 (*TIMP*-3) is reduced or absent in many cancers, and this loss of expression is regulated by methylation [59–61]. The product of this gene inhibits angiogenesis and cell migration and, therefore, is linked with the development of metastases. Accordingly, methylation of *TIMP*-3 appears to be a late event in HCC. Methylation of *TIMP*-3 in HCC is most often reported in advanced stage HCC, especially with extension into the portal vein [45,62].

Tissue factor pathway inhibitor-2 (*TFPI*-2) belongs to a family of genes that are apparently predisposed to aberrant methylation, especially in cancers [63]. By inhibiting the actions of metalloproteinases, *TFPI*-2 represses cellular invasion and migration. *TFPI*-2 hypermethylation with subsequent loss of protein expression is reported in approximately 50% of HCC cases, but not in adjacent, noncancerous tissue [64].

DNA repair

Mechanisms that proofread or repair DNA are frequently abnormal in cancer, resulting in mutations and microsatellite instability. Errors in DNA replication are corrected by the mismatch repair system (MMR). Promoter methylation and inactivation of the family of MMR enzymes (hMLH1, hMSH2 and hMSH3) is commonly detected in HCC, with methylation frequencies ranging from 15 to 75% [65,66]. This aberrant methylation is also found in cirrhotic tissue neighboring tumors, suggesting that defective MMR systems via methylation are a key early step in the development of HCC [54].

O^6-methylguanine DNA methyltransferase (*MGMT*) is the most abundant DNA repair gene in the liver [67]. Promoter hypermethylation of *MGMT* is found in up to 40% of HCC, particularly in those cancers associated with viral liver disease [66,68]. The functional relationship between viral disease and *MGMT* methylation is not clear. MGMT normally protects and repairs DNA from cytotoxic events that result in the alkylation of gaunine [54].

Glutithione *S*-transferase P1 (GSTP1) is an enzyme that prevents cellular damage caused by electrophilic and oxidative stress, and the inactivation of GSTP1 is potentially a crucial step in the pathogenesis of HCC [45,69]. Promoter methylation of *GSTP1* is common in both HCC tissue (89% of tumors) and serum of patients with HCC (50% of patients), indicating that GSTP1 may also serve as an effective biomarker for HCC [69].

CpG island methylator phenotype

The CIMP was originally described as an alternative pathway for colorectal carcinogenesis. CIMP-positive colon cancers exhibit hypermethylation of multiple genes, as well as microsatellite instability, and have distinct clinical, pathological and genetic features that differentiate them from sporadic colon cancers [21]. CIMP is also detected in other gastrointestinal malignancies, including HCC. A recent comprehensive analysis of methylation in HCC found that tumors with β-catenin mutations had significantly more methylation than cancers with *TP53* mutations [70]. Further analysis of a focused panel of hypermethylated genes in noncancerous tissue and HCC determined that methylation frequencies segregated into three groups. Group 1 was associated with advanced age, and included the genes *HIC-1*, *CASP8*, *GSTP1*, *SOCS1*, *RASSFIA*, *p16* and *APC*. The genes in group 2 were *CDH1*, *RUNX3*, *RIZ1*, *SFRP2* and *MINT31*, and these were associated with chronic viral hepatitis. The third group was cancer specific, and included *COX2*, *MINT1*, *CACNA1G*, *RASSF2*, *MINT2*, *Reprimo* and *DCC* [70]. This study is of particular importance because it characterizes the methylation status of multiple genes in a large cohort of samples with varying clinical presentations, rather than focusing on a specific gene. The findings also underscore the impact of aging and viral hepatitis on the pathogenesis of HCC, and perhaps target hepatitis C as a causative agent for the CIMP [71].

Histone modification

In comparison with hypermethylation, there are relatively few studies describing the specific modifications to histones that are involved in the development of liver cancer. For the most part, HDAC inhibitors are used in the laboratory to reverse genes that are silenced by epigenetic mechanisms in cancerous cell lines. One important group of proteins that are involved in histone modifications are the metastatic tumor antigens (MTAs) 1, 2 and 3. MTA2 has been reported to interact with TP53 and inhibit p53-mediated apoptosis by deacetylation. MTA2 is detected in more than 90% of a large cohort (n = 506) of human HCC samples. Notably, the MTA2 expression level strongly increased depending on the size and differentiation of HCC [72]. In a rodent model of dietary methyl deficiency that results in HCC, a progressive decrease in histone H4 lysine 20 tri-methylation and a gradual increase in Suv4–20h2 histone methyltransferase was detected in liver tumors. Moreover, a prominent increase in histone H3 lysine 9 trimethylation and in the expression of Suv39h1 histone methyltransferase was observed in preneoplastic lesions [73]. These findings indicate that certain histone modifications occur early and are crucial to the development of HCC.

Cholangiocarcinoma

Cholangiocarcinomas are tumors arising from the intra- and extrahepatic biliary epithelium. The molecular pathogenesis of CC is poorly understood. The etiology of CC is usually related to chronic biliary inflammation, which can be seen in primary sclerosing cholangitis, or infestation with liver flukes. Hepatitis C also appears to have an etiologic role [3]. As with most cancer, the neoplastic development of CC is thought to occur as a multistep pathway with the accumulation of genetic and epigenetic alterations. As described in HCC, multiple cellular pathways are affected by methylation in CC. For example, $p16^{INK4A}$ methylation and associated inactivation is detected in nearly 85% of CC. The methylation of $p16^{INK4A}$ is also detected in preneoplastic lesions, indicating that $p16^{INK4A}$ methylation is an early event in cholangiocarcinogenesis.

The short (p) arm of chromosome 3 is a region that is particularly susceptible to alterations in cancer. Several important tumor suppressor genes are located on the 3p arm, and are candidates of frequent epigenetic inactivation. Notably, RASSF1A, which inhibits cell cycle progression by blocking the action of cyclin D1 and negatively regulates mitosis, is methylated in 67% of CC cases. In addition, SEMA3B, which induces apoptosis in lung cancer cells, is silenced by hypermethylation in 100% of CC, although this was reported in a small number of tumors (n = 15) [74].

Additional genes that are reported to be methylated in CC are $p14^{ARF}$, *MGMT*, *CDH1*, *DAPK* and *GSTP1*. These genes are recognized as tumor suppressor genes, and the loss of their expression is associated with increased cell proliferation, increased cell migration, decreased ability of DNA to repair itself and the loss of apoptosis. However, the hypermethylation of these genes is not extremely common, typically being reported in less than 40% of tumors [75]. There is increasing evidence supporting the role of miRNA species in the pathogenesis of CC [76]. Although epigenetic inactivation of miRNA is described, it is not clear if this is a significant event in CC.

Conclusion

Epigenetic events that regulate gene expression have clearly emerged as a fundamental mechanism in the pathogenesis of hepatobiliary malignancies. Multiple genes that affect numerous cellular pathways are silenced by hypermethylation in HCC, and studying these genes has increased our understanding of how liver cancer develops and progresses. However, the majority of studies focus on methylation of a single gene or panel of genes in HCC, without

detailed investigation of the functional relevance of the gene silencing. With the advent of genome-wide microarray platforms, the 'methylome' of hepatobiliary cancers will be further defined. Currently, clinical decision-making for these cancers is based on the relatively crude assessment of tumor size and extension beyond the liver. Histology rarely plays a role, except for possibly predicting recurrence after resection. The molecular information gained from the epigenetic studies presented, in conjunction with other genetic information, could be used to develop a novel classification system for liver tumors. This would be of particular relevance in CC, where intrahepatic CC behaves differently to extrahepatic CC. This theoretical classification system could be designed to reflect so-called 'tumor biology' that could predict clinical outcomes, such as overall prognosis, risk of recurrence after surgery or response to chemotherapy. Certainly, future studies will need to be designed in a multicenter fashion so that the epigenetic behavior of liver tumors in different liver disease etiologies may be effectively collected and studied. In addition, prior to use in clinical practice, a consensus decision must be made on the optimal method by which to detect and report levels of methylation. The NIH (MD, USA) has released announcements in order to attract new grant applications to study epigenetic events in cancer, and this can only help to increase our scientific knowledge regarding these tumors. Finally, the majority of the published studies focus on methylation in the promoter region of genes. Novel discoveries have revealed significant and relevant levels of methylation affecting gene expression in regions of the genome not associated with the promoters. In the near future, these vast regions, dubbed 'CpG island shores' [77], are likely to be studied in hepatobiliary cancers, thus leading to new and exciting discoveries regarding the pathogenesis of these malignancies.

Future perspective

Therapeutic strategies

The important discoveries highlighted in this review have shed light on the pathogenesis of HCC and CC. Naturally, with this increased understanding, there is hope for the development of a new era of novel therapeutic agents that may effectively treat patients with these terrible malignancies. The beauty of epigenetic modifications to DNA is that they are potentially preventable or reversible. For example, blocking DNA methylation by inhibiting DNA methyltransferases results in demethylation of CpG islands in daughter cells, with subsequent re-expression of tumor suppressor genes and abrogation of tumor growth. There are several DNA demethylating compounds that are actively being investigated, including 5-azacytidine (AzaC), 5-aza-2′-deoxycytidine, procainamide and procaine [34]. It is important to recognize that many patients with advanced, unresectable or untransplantable HCC will have severe liver dysfunction, which limits drug tolerability. Indeed, toxicity has been a major limiting feature of these medications, and they are currently only indicated in patients with advanced myelodysplastic syndromes [71].

One interesting preclinical study combined sorafenib and rapamycin for the treatment of HCC [51]. As discussed, alterations to the RAS signaling pathway are inherent to the development and progression of HCC. Sorafenib was approved for the treatment of advanced HCC after the success of the Phase III Sorafenib HCC Randomized Assessment Protocol (SHARP) trial [78]. Sorafenib inhibits the actions of multiple kinases, including Raf-1, B-Raf, PDGFR-α and VEGFR-2 [79]. Rapamycin is an mTOR inhibitor and is commonly used as an immunosuppressant after solid organ transplantation. The mTOR signaling pathway is also involved in hepatocarcinogenesis [80]. This study demonstrated that through the blockade of the RAS and mTOR pathways, cell growth and proliferation was abrogated and apoptosis was induced. This study not only highlighted the epigenetic events that lead to RAS activation in HCC but also demonstrates that effective future treatments may require targeting of both genetic and epigenetic mechanisms [51]. In an additional study that targeted the epigenetic events that stimulate RAS activation in HCC, zebularine, a novel demethylating agent thought

to be much less toxic than AzaC, was demonstrated to induce apoptosis in HCC cell lines by reversing hypermethylation of *RASSF1A* and *NORE1A* [81]. These highlighted studies, in which multiple molecular pathways are targeted, set the stage and serve as examples for future clinical trials in the treatment of hepatobiliary malignancies.

Rather than systemically administered therapeutics, specialized delivery systems that specifically target aberrant methylation in tumors are actively being developed and studied. Abnormal promoter methylation has also been demonstrated to correlate with chemotherapy and radiation resistance [59]. In the future, it is conceivable that demethylating agents such as those presented could be used to enhance the effectiveness of traditional chemotherapy in HCC or CC [71].

There are also a host of HDAC inhibitors that are being studied for the treatment of cancer. Examples of these HDAC inhibitors include suberoylanilide hydroxamic acid, trichostatin A, valproic acid and sodium butyrate [34]. These agents result in an increase of histone acetylation by blocking the action of multiple HDACs, and are commonly used in laboratory experiments to reverse epigenetically induced gene silencing. There are also several early-phase clinical trials combining conventional therapy (sorafenib) with novel HDAC inhibitors, namely 4SC-201 and LBH589, for the treatment of HCC [4].

Biomarker development

Many of the epigenetic events described in this review have been detected in premalignant tissue, as well as in cancers. In addition, several of the frequently methylated genes can be detected in the serum of patients prior to or concurrent with the diagnosis of cancer. For this reason, DNA methylation, hypomethylation or histone modification are attractive candidates for early-detection biomarkers. An advantage for employing DNA hypermethylation (or hypomethylation) as biomarker of disease is the stability of DNA. Nevertheless, several important issues still remain that should be addressed; one of which is organ specificity, as many of the genes have aberrant promoter hypermethylation in other cancers. The second issue is the source of the biomarker. Obtaining bile or tumor biopsy specimens may be a challenge in some clinical scenarios, and with regards to diagnostic applicability, it is unlikely that epigenetic information will be more useful than histology. However, several studies have correlated epigenetic events with overall prognosis, risk of recurrence and response to therapy. Thus, it is conceivable that epigenetic data could be used to tailor therapy in the future [33]

In summary, the current benefit of epigenetic studies that are involved in hepatobiliary malignancies is that we have gained a better understanding of the processes that drive liver carcinogenesis. Although epigenetic events are readily reversible in the laboratory, it remains to be seen if this will be feasible in man. If epigenetic alterations are to be the target of novel therapeutic compounds, used as early-detection biomarkers, or to prognosticate patient outcomes, large-scale prospective data will need to be generated before this information will definitively translate into clinical practice.

Executive summary

- Epigenetic modification of DNA is a key feature in the pathogenesis of hepatobiliary malignancies.
- Pathways adversely affected by epigenetic alterations include apoptosis, cell cycle progression, DNA repair and cellular invasion and migration.
- Epigenetic events are potential candidates for biomarkers of early detection, prognosis and response to therapy.

- Epigenetic events are reversible, and are therefore sensible and rational targets for novel therapeutic approaches.

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Bibliography

Papers of special note have been highlighted as: ■ of interest

1. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557–2576. [PubMed: 17570226]
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–1917. [PubMed: 14667750]
3. El-Serag HB, Engels EA, Landgren O, et al. Risk of hepatobiliary and pancreatic cancers after hepatitis C virus infection: a population-based study of US veterans. Hepatology 2009;49:116–123. [PubMed: 19085911]
4■. Esteller M. Epigenetics in cancer. N Engl J Med 2008;358:1148–1159. Thorough and detailed review of the epigenetic mechanisms involved in cancer. Written by an international leader with unquestioned expertise in the field. [PubMed: 18337604]
5■. Boyault S, Rickman DS, de Reynies A, et al. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology 2007;45:42–52. Comprehensive investigation that reveals the molecular underpinnings of the complex interplay between genetics and epigenetics involved in hepatocellular carcinoma (HCC). [PubMed: 17187432]
6. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. Carcinogenesis 2010;31:27–36. [PubMed: 19752007]
7. Selaru FM, David S, Meltzer SJ, Hamilton JP. Epigenetic events in gastrointestinal cancer. Am J Gastroenterol 2009;104:1910–1912. [PubMed: 19661933]
8■. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–2054. Well-written and comprehensive review of epigenetic mechanisms and how they regulate gene transcription. Written by two internationally recognized experts in the field. [PubMed: 14627790]
9. Bestor TH. Transposons reanimated in mice. Cell 2005;122:322–325. [PubMed: 16096053]
10. Feinberg AP, Cui H, Ohlsson R. DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. Semin Cancer Biol 2002;12:389–398. [PubMed: 12191638]
11. Reik W, Lewis A. Co-evolution of X-chromosome inactivation and imprinting in mammals. Nat Rev Genet 2005;6:403–410. [PubMed: 15818385]
12. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 1983;301:89–92. [PubMed: 6185846]
13. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. Nat Rev Genet 2006;7:21–33. [PubMed: 16369569]
14. Wilson AS, Power BE, Molloy PL. DNA hypomethylation and human diseases. Biochim Biophys Acta 2007;1775:138–162. [PubMed: 17045745]
15. Ehrlich M. DNA methylation in cancer: too much, but also too little. Oncogene 2002;21:5400–5413. [PubMed: 12154403]
16. Fraga MF, Herranz M, Espada J, et al. A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. Cancer Res 2004;64:5527–5534. [PubMed: 15313885]
17. Fraga MF, Esteller M. Epigenetics and aging: the targets and the marks. Trends Genet 2007;23:413–418. [PubMed: 17559965]

18. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996;93:9821–9826. [PubMed: 8790415]

19. Dupont JM, Tost J, Jammes H, Gut IG. *De novo* quantitative bisulfite sequencing using the pyrosequencing technology. Anal Biochem 2004;333:119–127. [PubMed: 15351288]

20. Weber M, Davies JJ, Wittig D, et al. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 2005;37:853–862. [PubMed: 16007088]

21. Toyota M, Ho C, Ahuja N, et al. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res 1999;59:2307–2312. [PubMed: 10344734]

22. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. Cell 2007;128:669–681. [PubMed: 17320505]

23. Ballestar E, Paz MF, Valle L, et al. Methyl-CpG binding proteins identify novel sites of epigenetic inactivation in human cancer. EMBO J 2003;22:6335–6345. [PubMed: 14633992]

24■. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–692. Authoritative review detailing the epigenetic events commonly involved in carcinogenesis. [PubMed: 17320506]

25. Ma X, Ezzeldin HH, Diasio RB. Histone deacetylase inhibitors: current status and overview of recent clinical trials. Drugs 2009;69:1911–1934. [PubMed: 19747008]

26■. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007;8:286–298. Another excellent review, with much more detail about histone modifications than most other articles. [PubMed: 17339880]

27. Yang HI, Lu SN, Liaw YF, et al. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med 2002;347:168–174. [PubMed: 12124405]

28. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology 2005;42:1208–1236. [PubMed: 16250051]

29. Hsu YS, Chien RN, Yeh CT, et al. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology 2002;35:1522–1527. [PubMed: 12029639]

30. Soini Y, Chia SC, Bennett WP, et al. An aflatoxin-associated mutational hotspot at codon 249 in the *p53* tumor suppressor gene occurs in hepatocellular carcinomas from Mexico. Carcinogenesis 1996;17:1007–1012. [PubMed: 8640905]

31. Yam JW, Wong CM, Ng IO. Molecular and functional genetics of hepatocellular carcinoma. Front Biosci (Schol Ed) 2010;2:117–134. [PubMed: 20036934]

32. Baylin SB. Mechanisms underlying epigenetically mediated gene silencing in cancer. Semin Cancer Biol 2002;12:331–337. [PubMed: 12191632]

33■. Calvisi DF, Ladu S, Gorden A, et al. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. J Clin Invest 2007;117:2713–2722. Well-designed, genome-wide study investigating methylation and its prognostic significance in HCC. [PubMed: 17717605]

34. Roberts LR, Gores GJ. Hepatocellular carcinoma: molecular pathways and new therapeutic targets. Semin Liver Dis 2005;25:212–225. [PubMed: 15918149]

35. Lee HS, Kim BH, Cho NY, et al. Prognostic implications of and relationship between CpG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. Clin Cancer Res 2009;15:812–820. [PubMed: 19188151]

36. Lee JS, Chu IS, Heo J, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. Hepatology 2004;40:667–676. [PubMed: 15349906]

37. Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. Hepatology 2008;48:2047–2063. [PubMed: 19003900]

38. Ying Y, Tao Q. Epigenetic disruption of the WNT/β-catenin signaling pathway in human cancers. Epigenetics 2009;4:307–312. [PubMed: 19633433]

39. Csepregi A, Rocken C, Hoffmann J, et al. APC promoter methylation and protein expression in hepatocellular carcinoma. J Cancer Res Clin Oncol 2008;134:579–589. [PubMed: 17973119]

40. Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 2001;1:222–231. [PubMed: 11902577]
41. Liew CT, Li HM, Lo KW, et al. High frequency of *p16*^{INK4A} gene alterations in hepatocellular carcinoma. Oncogene 1999;18:789–795. [PubMed: 9989830]
42. Matsuda Y, Ichida T, Matsuzawa J, et al. p16(^{INK4}) is inactivated by extensive CpG methylation in human hepatocellular carcinoma. Gastroenterology 1999;116:394–400. [PubMed: 9922321]
43. Jin M, Piao Z, Kim NG, et al. p16 is a major inactivation target in hepatocellular carcinoma. Cancer 2000;89:60–68. [PubMed: 10897001]
44■. Roncalli M, Bianchi P, Bruni B, et al. Methylation framework of cell-cycle gene inhibitors in cirrhosis and associated hepatocellular carcinoma. Hepatology 2002;36:427–432. Thorough investigation of how methylation-induced derangements in the cell cycle contribute to the development of HCC. [PubMed: 12143052]
45. Lee S, Lee HJ, Kim JH, et al. Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. Am J Pathol 2003;163:1371–1378. [PubMed: 14507645]
46. Zhang YJ, Rossner P Jr, Chen Y, et al. Aflatoxin B1 and polycyclic aromatic hydrocarbon adducts, p53 mutations and p16 methylation in liver tissue and plasma of hepatocellular carcinoma patients. Int J Cancer 2006;119:985–991. [PubMed: 16570275]
47. Zhang YJ, Wu HC, Shen J, et al. Predicting hepatocellular carcinoma by detection of aberrant promoter methylation in serum DNA. Clin Cancer Res 2007;13:2378–2384. [PubMed: 17438096]
48. Matsuda Y. Molecular mechanism underlying the functional loss of cyclin-dependent kinase inhibitors p16 and p27 in hepatocellular carcinoma. World J Gastroenterol 2008;14:1734–1740. [PubMed: 18350604]
49. Jung JK, Arora P, Pagano JS, Jang KL. Expression of DNA methyltransferase 1 is activated by hepatitis B virus X protein via a regulatory circuit involving the p16^{INK4a}–cyclin D1–CDK 4/6–pRb–E2F1 pathway. Cancer Res 2007;67:5771–5778. [PubMed: 17575144]
50. Wong IH, Lo YM, Yeo W, Lau WY, Johnson PJ. Frequent p15 promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients. Clin Cancer Res 2000;6:3516–3521. [PubMed: 10999738]
51■. Newell P, Toffanin S, Villanueva A, et al. RAS pathway activation in hepatocellular carcinoma and anti-tumoral effect of combined sorafenib and rapamycin *in vivo*. J Hepatol 2009;51:725–733. Cutting edge preclinical study using two well known agents to target genetic and epigenetic abnormalities in HCC. [PubMed: 19665249]
52. Yea S, Narla G, Zhao X, et al. RAS promotes growth by alternative splicing-mediated inactivation of the KLF6 tumor suppressor in hepatocellular carcinoma. Gastroenterology 2008;134:1521–1531. [PubMed: 18471523]
53. Yasuda E, Kumada T, Takai S, et al. Attenuated phosphorylation of heat shock protein 27 correlates with tumor progression in patients with hepatocellular carcinoma. Biochem Biophys Res Commun 2005;337:337–342. [PubMed: 16199221]
54. Tischoff I, Tannapfe A. DNA methylation in hepatocellular carcinoma. World J Gastroenterol 2008;14:1741–1748. [PubMed: 18350605]
55. Yu J, Ni M, Xu J, et al. Methylation profiling of twenty promoter-CpG islands of genes which may contribute to hepatocellular carcinogenesis. BMC Cancer 2002;2:29. [PubMed: 12433278]
56. Nomoto S, Kinoshita T, Kato K, et al. Hypermethylation of multiple genes as clonal markers in multicentric hepatocellular carcinoma. Br J Cancer 2007;97:1260–1265. [PubMed: 17968429]
57. Harder J, Opitz OG, Brabender J, et al. Quantitative promoter methylation analysis of hepatocellular carcinoma, cirrhotic and normal liver. Int J Cancer 2008;122:2800–2804. [PubMed: 18351580]
58. Kwon GY, Yoo BC, Koh KC, Cho JW, Park WS, Park CK. Promoter methylation of E-cadherin in hepatocellular carcinomas and dysplastic nodules. J Korean Med Sci 2005;20:242–247. [PubMed: 15831995]
59. Hamilton JP, Sato F, Greenwald BD, et al. Promoter methylation and response to chemotherapy and radiation in esophageal cancer. Clin Gastroenterol Hepatol 2006;4:701–708. [PubMed: 16678495]
60. Wang Y, Zhang D, Zheng W, Luo J, Bai Y, Lu Z. Multiple gene methylation of nonsmall cell lung cancers evaluated with 3-dimensional microarray. Cancer 2008;112:1325–1336. [PubMed: 18286531]

61. Hoque MO, Begum S, Brait M, et al. Tissue inhibitor of metalloproteinases-3 promoter methylation is an independent prognostic factor for bladder cancer. J Urol 2008;179:743–747. [PubMed: 18082200]
62. Lu GL, Wen JM, Xu JM, Zhang M, Xu RB, Tian BL. Relationship between TIMP-3 expression and promoter methylation of *TIMP-3* gene in hepatocellular carcinoma. Zhonghua Bing Li Xue Za Zhi 2003;32:230–233. [PubMed: 12882688]
63. Glockner SC, Dhir M, Yi JM, et al. Methylation of TFPI2 in stool DNA: a potential novel biomarker for the detection of colorectal cancer. Cancer Res 2009;69:4691–4699. [PubMed: 19435926]
64. Wong CM, Ng YL, Lee JM, et al. Tissue factor pathway inhibitor-2 as a frequently silenced tumor suppressor gene in hepatocellular carcinoma. Hepatology 2007;45:1129–1138. [PubMed: 17464989]
65. Park JH, Cho SB, Lee WS, et al. Methylation pattern of DNA repair genes and microsatellite instability in hepatocellular carcinoma. Korean J Gastroenterol 2006;48:327–336. [PubMed: 17132921]
66. Matsukura S, Soejima H, Nakagawachi T, et al. CpG methylation of *MGMT* and hMLH1 promoter in hepatocellular carcinoma associated with hepatitis viral infection. Br J Cancer 2003;88:521–529. [PubMed: 12592365]
67. Gerson SL, Trey JE, Miller K, Berger NA. Comparison of O_6-alkylguanine-DNA alkyltransferase activity based on cellular DNA content in human, rat and mouse tissues. Carcinogenesis 1986;7:745–749. [PubMed: 3698202]
68. Su PF, Lee TC, Lin PJ, et al. Differential DNA methylation associated with hepatitis B virus infection in hepatocellular carcinoma. Int J Cancer 2007;121:1257–1264. [PubMed: 17534893]
69. Wang J, Qin Y, Li B, Sun Z, Yang B. Detection of aberrant promoter methylation of GSTP1 in the tumor and serum of Chinese human primary hepatocellular carcinoma patients. Clin Biochem 2006;39:344–348. [PubMed: 16527261]
70■. Nishida N, Nagasaka T, Nishimura T, Ikai I, Boland CR, Goel A. Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. Hepatology 2008;47:908–918. Detailed, genome-wide study that categorizes epigenetic events into categories associated with aging, cancer and viral disease. [PubMed: 18161048]
71. Boland CR, Shin SK, Goel A. Promoter methylation in the genesis of gastrointestinal cancer. Yonsei Med J 2009;50:309–321. [PubMed: 19568590]
72. Lee H, Ryu SH, Hong SS, et al. Overexpression of metastasis-associated protein 2 is associated with hepatocellular carcinoma size and differentiation. J Gastroenterol Hepatol 2009;24:1445–1450. [PubMed: 19702911]
73. Pogribny IP, Ross SA, Tryndyak VP, Pogribna M, Poirier LA, Karpinets TV. Histone H3 lysine 9 and H4 lysine 20 trimethylation and the expression of Suv4–20h2 and Suv–39h1 histone methyltransferases in hepatocarcinogenesis induced by methyl deficiency in rats. Carcinogenesis 2006;27:1180–1186. [PubMed: 16497704]
74. Tischoff I, Markwarth A, Witzigmann H, et al. Allele loss and epigenetic inactivation of 3p21.3 in malignant liver tumors. Int J Cancer 2005;115:684–689. [PubMed: 15704097]
75. Tischoff I, Wittekind C, Tannapfel A. Role of epigenetic alterations in cholangiocarcinoma. J Hepatobiliary Pancreat Surg 2006;13:274–279. [PubMed: 16858537]
76. Selaru FM, Olaru AV, Kan T, et al. MicroRNA-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3. Hepatology 2009;49:1595–1601. [PubMed: 19296468]
77. Doi A, Park IH, Wen B, et al. Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. Nat Genet 2009;41:1350–1353. [PubMed: 19881528]
78. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–390. [PubMed: 18650514]
79. Liu L, Cao Y, Chen C, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66:11851–11858. [PubMed: 17178882]
80. Villanueva A, Chiang DY, Newell P, et al. Pivotal role of mTOR signaling in hepatocellular carcinoma. Gastroenterology 2008;135:1972–1983. 1983 E1–E11. [PubMed: 18929564]

81. Calvisi DF, Ladu S, Gorden A, et al. Ubiquitous activation of RAS and JAK/STAT pathways in human HCC. Gastroenterology 2006;130:1117–1128. [PubMed: 16618406]