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Review

Aberrant DNA Methylation in Cholangiocarcinoma

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Abstract: Cholangiocarcinoma is an epithelial malignancy arising in the region between the intrahepatic bile ducts and the ampulla of Vater at the distal end of the common bile duct. The effect of current chemotherapy regimens against cholangiocarcinoma is limited, and the prognosis of patients with cholangiocarcinoma is poor. Aberrant DNA methylation and histone modification induce silencing of tumor suppressor genes and chromosomal instability during carcinogenesis. Studies have shown that the tumor suppressor genes and microRNAs (miRNAs) including *MLH1*, *p14*, *p16*, *DAPK*, *miR-370 and miR-376c* are frequently methylated in cholangiocarcinoma. Silencing of these tumor suppressor genes and miRNAs plays critical roles in the initiation and progression of cholangiocarcinoma. In addition, recent studies have demonstrated that DNA methylation inhibitors induce expression of endogenous retroviruses and exert the anti-tumor effect of via an anti-viral immune response. Aberrant DNA methylation of tumor suppressor genes and miRNAs could be a powerful biomarker for diagnosis and treatment of cholangiocarcinoma. Epigenetic therapy with DNA methylation inhibitors hold considerable promise for the treatment of cholangiocarcinoma through re-activation of tumor suppressor genes and miRNAs as well as induction of an anti-viral immune response.

Keywords: Cholangiocarcinoma; DNA methylation; Tumor suppressor gene; microRNA; DNA methylation inhibitor

Introduction

Cholangiocarcinoma is an epithelial malignancy arising in the region between the intrahepatic bile ducts and the ampulla of Vater at the distal end of the common bile duct [1, 2]. The number of cholangiocarcinoma patients is apparently increasing, and five-year survival rates are approximately 20%, as most of patients are diagnosed at an advanced stage. Currently, patients with cholangiocarcinoma receive chemotherapy regimens including cisplatin and gemcitabine. However, the effect of these chemotherapies is limited, and development of new therapeutic strategy against cholangiocarcinoma is necessary [3].

Epigenetics is an acquired modification of methylation and/or acetylation of chromatin DNA or histone proteins, which regulates downstream gene expression without an alteration in the DNA sequence itself [4]. Epigenetic alterations can be induced by aging, chronic inflammation, or viral infection. Aberrant DNA methylation and histone modification induce silencing of tumor suppressor genes and chromosomal instability and play critical roles in the initiation and progression of various cancers [5-7]. MicroRNAs (miRNAs) are small non-coding RNAs that function as endogenous silencers of numerous target genes. Hundreds of miRNAs have been identified in the human genome. miRNAs are expressed in a tissue-specific manner and play important roles in cell proliferation, apoptosis, and differentiation. We and other groups have revealed that epigenetic alterations regulate not only protein-coding genes but also non-coding genes such as miRNAs in cancer cells [8-10].

Chromatin-modifying drugs such as DNA methylation inhibitors and histone deacetylase (HDAC) inhibitors have clinical promise for cancer therapy [4, 11]. The DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-Aza-CdR) and the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) are emerging as a promising agent for epigenetic therapy of human malignancies [12, 13]. Aberrant DNA methylation at CpG island promoters of tumor suppressor genes is frequently

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observed in various human malignancies including cholangiocarcinoma. The DNA methylation inhibitor 5-Aza-CdR, which is an analog of cytidine, has been widely studied and was recently approved for the treatment of myelodysplastic syndrome (MDS). However the effect of DNA methylation inhibitors on patients with cholangiocarcinoma remains to be elucidated. In this review, we summarize the current knowledge regarding aberrant DNA methylation of important tumor suppressor genes and miRNAs in cholangiocarcinoma as well as effects of DNA methylation inhibitors on cholangiocarcinoma.

Aberrant DNA methylation as a biomarker of cholangiocarcinoma

Malignant tumors developing in the biliary tract are difficult to diagnose at an early stage because of their anatomical locations. In addition, useful biomarkers for biliary tract cancers have not been developed. Most of cholangiocarcinoma patients are diagnosed at an advanced stage, and aggressive cancers easily infiltrate surrounding organs and become unresectable. Early detection of cholangiocarcinoma might improve the prognosis of patients, and development of useful biomarkers of cholangiocarcinoma would be beneficial for prompt and more effective treatment. One of the most powerful biomarkers in cancer is DNA methylation of tumor suppressor genes. We summarized genes frequently methylated in cholangiocarcinoma in Table 1.

MLH1 protein is one component of a system of seven DNA mismatch repair (MMR) proteins that work coordinately in sequential steps to initiate repair of DNA mismatches in humans. Several studies have demonstrated that DNA hypermethylation on the promoter region of the *hMLH1* gene is associated with poor prognosis of patients with cholangiocarcinoma [14, 15]. The *DCLK1*, *CDO1*, *ZSCAN18* and *ZNF331* genes have been identified as novel biomarkers of colorectal cancers, and these genes are frequently methylated across gastrointestinal cancers including cholangiocarcinoma [16]. Negative correlation between promoter DNA methylation and gene expression has been observed for the *DCLK1*, *CDO1*, *ZSCAN18* and *ZNF331* genes, suggesting that aberrant DNA methylation of these genes indicates epigenetic similarities among gastrointestinal cancers such as colon, pancreatic and bile duct cancer. The *INK4a-ARF* (*CDKN2A*) locus on chromosome 9p21 encodes two tumor suppressor proteins, p16 (INK4a) and p14 (ARF), whose functions are inactivated in many human cancers. Recent studies have shown that *p16* (*INK4a*) and *p14* (*ARF*) are inactivated by DNA hypermethylation in cholangiocarcinoma, which may result in cell cycle dysregulation [17, 18]. Liu *et al*. have demonstrated that the *death-associated protein kinase* (*DAPK*) gene is suppressed by promoter hypermethylation in cholangiocarcinoma. Silencing of the *DAPK* gene by DNA hypermethylation results in resistance to apoptosis and immunological surveillance [19]. In addition, it has been reported that *p53* mutation combined with DNA methylation of the *DAPK*, *p14* (*ARF*) and *ASC* genes correlates with malignancy and poor prognosis of patients with chrangiocarcinoma [20].

Cancer cells are considered to be heterogeneous with a hierarchy of "stemness" in solid cancer tissues. Stem cells have the ability to perpetuate themselves through self-renewal and to generate mature cells of various tissues through differentiation. A subpopulation of cancer cells with distinct stem-like properties is responsible for tumor initiation, invasive growth, and metastasis formation, and these are defined as cancer stem cells [21]. As cancer stem cells are resistant to conventional chemotherapies and radiation therapy, in the context of cholangiocarcinoma it would be desirable to develop a therapeutic strategy specifically targeting cancer stem cells. Sriraksa *et al.* have reported that hypermethylation of multiple CpG sites of genes associated with a stem cell-like phenotype is a common molecular aberration in cholangiocarcinoma [22], indicating that aberrant DNA methylation plays a critical role role in "cancer stemness" of cholangiocarcinoma.

Early diagnosis is very important for patients with refractory cancers, but detection of cholangiocarcinoma at an early stage is still challenging because it is difficult to visualize biliary tract tumors by existing imaging modalities [23]. In order to overcome this problem, Shin *et al.* have developed a useful method for detection of cholangiocarcinoma cells using bile fluid [24]. This method involving DNA methylation assay consisting of a five-gene panel (*CCND2*, *CDH13*, *GRIN2B*, *RUNX3* and *TWIST1*) is able to detect cholangiocarcinoma cells with a sensitivity of 83%. Less invasive examinations such as this method using bile fluid are important for minimizing the burden

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on the patient. These studies have shown that detection of DNA methylation is a powerful diagnostic strategy for patients with cholangiocarcinoma.

Table 1. Genes frequently methylated in cholangiocarcinoma

Gene	Funtion	Sample	Reference
MLH1	DNA repair	tissue	14, 15
DCLK1	stemness	tissue	16
CDO1	growth	tissue	16
ZSCAN18	unknown	tissue	16
ZNF331	growth invasion	tissue	16
p14 (*ARF*)	cell cycle regulator	tissue	17,18, 20
p16 (*INK4a, CDKN2A*)	cell cycle regulator	tissue QBC939 cell line	17, 18, 20, 32, 33
DAPK	apoptosis	tissue QBC939 cell line	19, 20, 32
CCND2	growth	bile fluid	24
CDH13	growth invasion	bile fluid	24
GRIN2B	growth	bile fluid	24
RUNX3	growth differentiation	bile fluid	24
TWIST1	migration invasion	bile fluid	24
EGFR	growth	Mz-ChA-1 cell line	28
LKB1	growth migration invasion	tissue HuH-28 cell line RBE cell line SSP-25 cell line	29

DNA methylation inhibitors are promising therapeutic agents against cholangiocarcinoma

Chronic inflammation in the liver may contribute to malignant transformation of cholangiocytes [25]. It is assumed that persistent inflammation promotes carcinogenesis through DNA damage and tissue repair as well as activation of cytokines and other growth factors [26]. A previous study has demonstrated that cholangiocyte-derived cytokines, such as interleukin 6 (IL-6), transforming growth factor α (TGF-α) and tumor necrosis factor- α (TNF- α) regulate cholangiocyte intracellular signaling and promote carcinogenesis [27]. Figure. 1 shows the molecular mechanism underlying the initiation and progression of cholangiocarcinoma. When chronic inflammation and cholestasis arise due to liver injury, biliary epithelial cells release inflammation-associated cytokines such as IL-6 and TNF-α, which leads to accelerated growth of biliary epithelial cells. Accelerated proliferation of biliary epithelial cells promotes gene mutation and aberrant DNA methylation of tumor suppressor genes, leading to the initiation of cholangiocarcinoma. Wehbe *et al.* have previously reported that IL-6 contributes to the growth of cholangiocarcinoma cells through aberrant DNA methylation on the promoter region of tumor suppressor genes [28]. IL-6 decreased DNA methylation level on the promoter region of the *EGFR* gene, which leads to increased expression of the EGFR protein. These findings suggest that persistent cytokine stimulation in biliary epithelial cells could promote the initiation and progression of tumors via epigenetic alterations. Wang *et al.* have shown that suppression of the tumor suppressor *liver kinase B1* (*LKB1*) due to aberrant DNA methylation is associated with enhanced the Wnt signaling and malignant characteristics of human cholangiocarcinoma [29]. The expression of the *LKB1* gene was suppressed in cholangiocarcinoma tissues relative to adjacent normal tissues and knockdown of *LKB1* enhanced the growth, migration and invasion of tumors, along with activation of the Wnt signaling.

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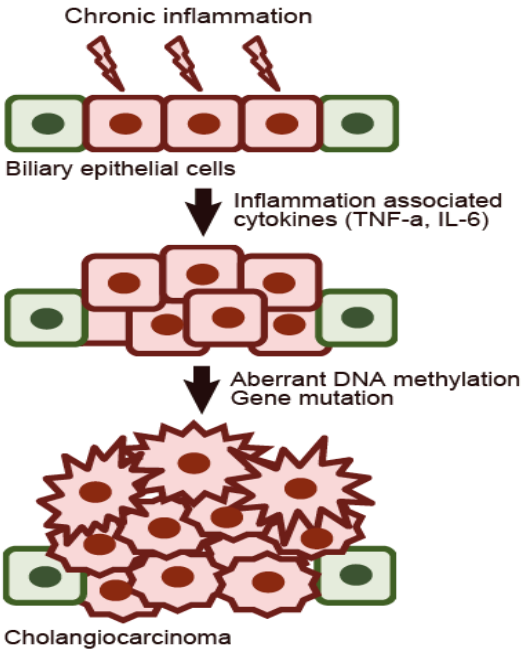

Figure 1. The molecular mechanism underlying the initiation of cholangiocarcinoma. When chronic inflammation and cholestasis arise due to liver injury, biliary epithelial cells release inflammation-associated cytokines such as IL-6 and TNF-α which leads to accelerated growth of biliary epithelial cells. Accelerated proliferation of biliary epithelial cells promotes gene mutation and aberrant DNA methylation of tumor suppressor genes, leading to the initiation of cholangiocarcinoma.

Figure. 2 shows a scheme for activation of tumor suppressor genes by inhibition of DNA methylation on their promoter regions. In cancer cells, tumor suppressor genes are silenced by DNA hypermethylation on CpG island promoter regions. DNA methylation inhibitors such as 5-Aza-CdR can re-activate epigenetically silenced tumor suppressor genes by inhibition of DNA methylation on promoter regions. Several studies have evaluated the effect of DNA methylation inhibitors on cholangiocarcinoma. The DNA methylation inhibitor zebularine inhibited human cholangiocarcinoma cells through alteration of DNA methylation status [30]. Zebularine exerted an anti-tumor effect on cholangiocarcinoma cells through suppression of DNA methyltransferases. Zebularine altered the DNA methylation status and suppressed the Wnt signaling pathway, resulting in decreased expression of CTNNB1. Several reports have indicated that tumor suppressor genes that were silenced in cholangiocarcinoma could be re-activated by the DNA methylation inhibitor 5-Aza-CdR [31, 32]. Liu *et al.* have reported that treatment of cholangiocarcinoma cells with 5-Aza-CdR inhibited cell growth and induced apoptosis by reactivation of p53-BAX mitchondrial apoptosis genes [32]. Xiang *et al.* have demonstrated that knockdown of the major DNA methyltransferase *DNMT1* restores the expression levels of tumor suppressor genes, which results in inhibition of the proliferation of cholangiocarcinoma cells [33]. These findings suggest that various tumor suppressor genes are inhibited by DNMT1-induced DNA hypermethylation in their promoter regions, which enhances proliferation, migration and invasion of cholangiocarcinoma cells. The biological effects of tumor suppressor genes frequently methylated in cholangiocarcinoma are summarized in Table 1. DNA methylation inhibitors such as 5-Aza-CdR and zebularine might have great promise for the treatment of cholangiocarcinoma. However, these DNA methylation inhibitors affect without gene specificity. Lee *et al.* have shown that human N-α-acetyltransferase 10 protein (hNaa10p) contributes to tumorigenesis by facilitating DNMT1-mediated tumor suppressor gene silencing [34]. They have confirmed that the oncogenic potential of hNaa10p depends on its interaction with DNMT1. hNaa10p positively regulates DNMT1 enzymatic activity by facilitating its binding to DNA and recruitment to the promoters of tumor suppressor genes such as E-cadherin. These data suggest that DNMT1-

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induced gene silencing may affect tumor suppressor genes rather than oncogenes in cancer cells. Further studies are necessary to develop DNA methylation inhibitors that specifically affect only the CpG island promoter region of tumor suppressor genes to reduce the side effects of epigenetic therapy.

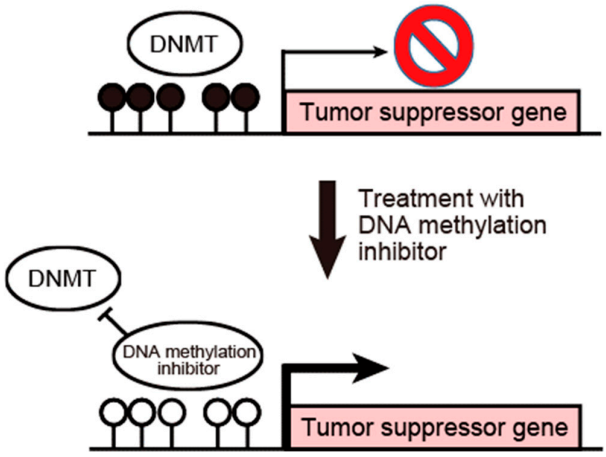

Figure. 2. Activation of tumor suppressor genes by inhibition of DNA methylation on their promoter regions. In cancer cells, tumor suppressor genes are silenced by DNA hypermethylation on CpG island promoter regions. DNA methylation inhibitors such as 5-Aza-CdR can re-activate epigenetically silenced tumor suppressor genes by inhibition of DNA methylation on promoter regions. Solid circle, methylated DNA; clear circle, unmethylated DNA.

Suppression of tumor suppressor miRNAs by DNA methylation in cholangiocarcinoma

miRNAs are small non-coding RNAs that function as silencers of various target genes and regulate cell growth and differentiation. Deregulation of miRNAs induces the initiation and progression of cancers by modifying their target tumor suppressor genes or oncogenes [35]. Braconi *et al.* have shown that IL-6 can regulate the activity of DNMT1 by miRNAs in cholangiocarcinoma cells [36]. They verified that *miR-148a* and *miR-152* regulate DNMT1 expression as their targets and showed that IL-6 can regulate the activity of DNMT1 and expression of DNA methylation-dependent tumor suppressor genes by modulation of *miR-148a* and *miR-152*. These findings provide a link between this inflammation-associated cytokine and oncogenesis in cholangiocarcinoma. In addition, several studies have shown that tumor suppressor miRNAs are regulated by DNA methylation. Meng *et al*. have reported that expression of DNA methyltransferases was increased by IL-6 overexpression and the tumor suppressor *miR-370* was inactivated by DNA methylation in cholangiocarcinoma cells [37]. The oncogene *mitogen-activated protein kinase kinase kinase 8* (*MAP3K8*) was identified as a target of *miR-370*. 5-Aza-CdR increased the expression of *miR-370* in malignant cells, while the expression in non-malignant cells was unchanged. Thus, IL-6 may contribute to tumor growth by modulation of expression of *miR-370* in cholangiocarcinoma cells. These findings define a mechanism by which inflammation-associated cytokines can epigenetically modulate gene expression and directly contribute to the initiation and development of cholangiocarcinoma.

Iwaki *et al*. have also shown that *miR-376c* was regulated by DNA methylation and associated with tumor suppression by targeting *growth factor receptor-bound protein 2* (*GRB2*) [38]. They found higher methylation levels of CpG sites upstream of the *miR-376c* gene in cholangiocarcinoma cells relative to normal intrahepatic biliary epithelial cells. The direct target genes and biological functions of miRNAs frequently methylated in cholangiocarcinoma are summarized in Table 2. Since miRNAs regulate several target genes including cancer-related genes, replacement of tumor suppressor miRNAs might have implications for the treatment of cholangiocarcinoma as well as activation of tumor suppressor miRNAs by epigenetic therapy using chromatin-modifying agents.

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Table 2. miRNAs frequently methylated in cholangiocarcinoma

miRNA	Target gene	Function	Sample	Reference
miR-370	MAP3K8	cell proliferation	MzChA-1 cell line KMCH-1 cell line	37
miR-376c	GRB2	migration	HuCCT1 cell line	38

Anti-tumor effect of DNA methylation inhibitors via an anti-viral immune response

Other anti-tumor effects of chromatin-modifying drugs have been demonstrated in cancers including colon cancer. One of these other anti-tumor effects is induction of tumor cell differentiation. A subpopulation of cancer cells with distinct stem-like properties is responsible for tumor initiation, invasive growth, and metastasis formation, and these are defined as cancer stem cells [21]. As cancer stem cells are resistant to conventional chemotherapies and radiation therapy, it would be desirable to develop a therapeutic strategy specifically targeting cancer stem cells. Hatano *et al.* have previously shown that DNA demethylation exerts a tumor-suppressive effect on colon cancers by inducing tumor differentiation [39]. They found that the promoter region of the *Caudal type homeobox 1* (*CDX1*) gene was methylated specifically in colon cancer cells. Upregulation of *CDX1* increased the expression of genes related to intestinal differentiation. This suggested that the promoters of transcriptional factor genes regulating cell differentiation were silenced by DNA hypermethylation in colon cancer cells to sustain their undifferentiated status.

Recent studies have proved that the major effect of DNA methylation inhibitors is to induce interferon-responsive genes by increasing double-stranded RNA (dsRNA) containing endogenous retrovirus (ERV) [40, 41]. Different ERV gene families constitute about eight percent of the human genome and are considered to be long terminal repeat [42] retrotransposons. Innate immune responses are activated by the expression of ERV-producing nucleic acids or proteins with viral signatures [43]. Roulois *et al.* have recently proposed that 5-Aza-CdR could be used to target colorectal cancer stem cells by inducing viral mimicry [40]. Their data suggested that induction of dsRNAs is derived at least in part from ERV elements, which activate the MDA5/MAVS RNA recognition pathway. Figure. 3 shows a scheme for activation of an anti-viral immune response induced by inhibition of DNA methylation. In a normal state, the 5′ long terminal repeat (LTR) sequences of ERVs are heavily methylated and expression of ERVs is silenced. When DNA methylation at the 5′ LTR sequences is inhibited by DNA methylation inhibitors, expression of ERVs is induced. Increased expression of dsRNAs derived from ERVs leads to induction of an anti-viral immune response such as activation of interferon-responsive genes.

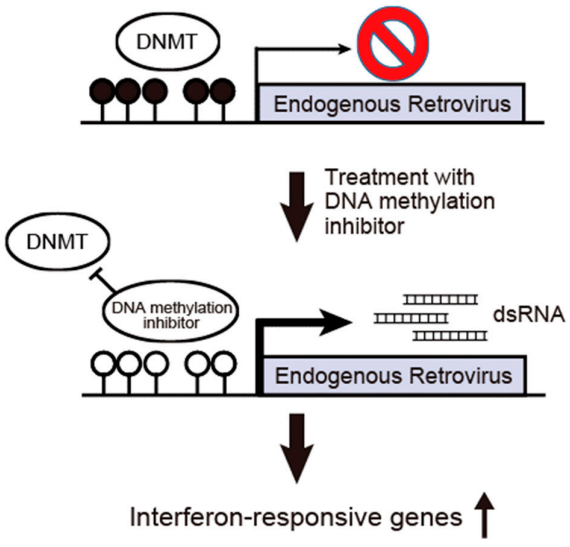

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Figure. 3. Activation of an anti-viral immune response induced by inhibition of DNA methylation. In a normal state, the 5′ LTR sequences of ERVs are heavily methylated and expression of ERVs is silenced. When DNA methylation at the 5′ LTR sequences is inhibited by DNA methylation inhibitors, expression of ERVs is induced. Increased expression of dsRNAs derived from ERVs leads to induction of an anti-viral immune response such as activation of interferon-responsive genes.

We have also reported that DNA methylation inhibition suppresses intestinal tumor organoids by inducing anti-viral response [44]. We have established tumor organoids derived from the *Apc*$^{min/+}$ mouse, a model of colon cancer, using a new 3D culture system that allows Lgr5-positive stem cells to form cyst-like structures (organoids) [45]. This organoid culture system closely recapitulates the properties of the original tumors, and is useful for drug screening and precision medicine [46]. We have demonstrated that 5-Aza-CdR shrinks intestinal tumor organoids derived from *Apc*$^{min/+}$ mice [44]. We have revealed that the expression of interferon-responsive genes such as *Irf7*, *Rig1* and *Mda5* was increased by DNA methylation inhibition in tumor organoids after 5-Aza-CdR treatment or *Dnmt1* knockdown. The expression of murine ERVs were significantly upregulated after treatment of tumor organoids with 5-Aza-CdR. These findings suggested that treatment with DNA methylation inhibitors to activate an innate immune response would be beneficial for patients with various types of cancers including cholangiocarcinoma. Wrangle *et al*. have shown that DNA methylation inhibitors can upregulate transcripts and protein of PD-L1, a key ligand mediator of immune tolerance [47]. Through analysis of samples from The Cancer Genome Atlas (TCGA), they also demonstrated that a significant proportion of primary non-small cell lung cancers (NSCLCs) have low expression of DNA methylation inhibitor-induced immune genes such as PD-L1. Their data suggest that combination of chromatin-modifying agents with immune checkpoint blockade therapies would activate the immune response of the host to cancer cells.

The development of anti-metabolite drugs that are dependent on the cell cycle of cancer cells has revealed a serious problem in that they also act on normal cells and normal stem cells. Therefore, molecular targeting therapeutic agents have been developed to avoid seriously damaging normal cells. One such molecular targeting therapeutic agent is herceptin, approved for the treatment of breast cancer. Although herceptin has improved the relapse-free survival of patients with breast cancer [48], it is still very difficult to eliminate the cancer completely, because cancers have various mutations and different forms of aberrant epigenetic status. In this respect, chromatin-modifying drugs have great promise for cancer therapy because modification of epigenetic status alone can inhibit various tumor characteristics such as proliferation, migration, invasion and dedifferentiation. It has been demonstrated that reprofiling of FDA-approved drugs in combination with chromatin-modifying drugs can be implemented into clinical trials for colon cancer [49].

In conclusion, aberrant DNA methylation of tumor suppressor genes and miRNAs could be a powerful biomarker for diagnosis and treatment of cholangiocarcinoma. Epigenetic therapy with DNA methylation inhibitors hold considerable promise for the treatment of cholangiocarcinoma through re-activation of tumor suppressor genes and miRNAs as well as induction of an anti-viral immune response.

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