Abstract. Cholangiocarcinoma (CCA) is a highly lethal malignant tumor arising from the biliary tract epithelium. Chronic inflammatory conditions, including primary sclerosing cholangitis, liver fluke infestation, and hepatolithiasis, are considered risk factors, but the cause is still unknown in most cases. Recent advances in molecular pathogenesis have highlighted the importance of epigenetic alterations, including promoter hypermethylation and histone deacetylation, in the process of cholangiocarcinogenesis. More recently, research interest has been focusing on microRNA (mir), a major subtype of non-coding RNA. Mir is highly conserved among species and regulates the expression of specific target genes by binding to the 3'-untranslated regions of messenger RNA. The number of studies on a possible link between mir and various cancers is growing. This review provides a comprehensive overview of the genes currently known to be hypermethylated in CCA and their putative roles in cholangiocarcinogenesis. The epigenetic role of mir in the pathogenesis of CCA is also discussed.

Contents
1. Introduction
2. Genetic alterations in CCA
3. Epigenetic alterations in CCA
4. Conclusions

1. Introduction

Cholangiocarcinoma (CCA) is a devastating neoplasm originating from cholangiocytes, the epithelial cells that line the biliary apparatus (1). It accounts for only 10-15% of hepatobiliary neoplasm (2,3). Two-thirds of CCAs involve the extrahepatic bile duct, whereas the remaining one-third affects the intrahepatic biliary tree (1,4). The disease is notoriously difficult to diagnose and is usually fatal because of its typically late clinical presentation and the lack of effective therapeutic modalities (1,4). Most patients have unresectable disease at presentation, and the overall survival rate, including resected patients, is poor, with less than 5% of patients surviving 5 years (5). Although CCA is a relatively rare tumor, interest in this disease is rising as the incidence and mortality rates are increasing worldwide (2,3). CCA is associated with chronic inflammatory conditions of the biliary system, including hepatolithiasis, liver fluke infestation, choledochal cysts, and primary sclerosing cholangitis (PSC) (1,4). However, for most CCA cases, the cause is unknown, and affected individuals have no history of exposure to, or association with, such risk factors (1,4).

At present, little is known about the molecular mechanisms in CCA. As for many other tumors, the development of CCA must be understood as a multistep process, with the accumulation of genetic and epigenetic alterations in regulatory genes, leading to the activation of oncogenes and the inactivation or loss of tumor suppressor genes (TSGs) (6). This review discusses the epigenetic inactivation of different TSGs in CCA. The limited data regarding microRNA (mir) regulation of cholangiocarcinogenesis are also summarized.

2. Genetic alterations in CCA

The milieu of chronic biliary inflammation, along with cholestasis, leads to the production of cytokines and reactive oxygen species, and this causes protracted cellular stress and irreversible DNA damage (1,2). As a result, cholangiocytes attain cellular phenotypes that result in malignant transformation (1). The proposed pathways that participate in cholangiocarcinogenesis include: self-sufficiency and proliferation; apoptosis resistance; escape from senescence; and tumor invasiveness and metastasis (1). Molecular mechanisms responsible for bile duct carcinogenesis likely include the interaction of genetic variants and somatic cell alterations (1,4,6). The genetic changes in CCA include: mutations of K-ras, p53, p16\(^{INK4a}\), and Smad4; loss of heterozygosity (LOH) of APC; and allelic losses on 3p13-p21 and 8q22 (7-11). Among the genetic abnormalities that have been demonstrated in CCA, p53 mutations and activating K-ras mutations are the most frequent (6). Nevertheless, the
reported rates of genetic alterations in CCA vary widely among studies (4,6). These variations are presumably due to
differences in the subsites of cancers, racial and geographical
variations in the study populations, and the use of different
 assay techniques (4).

3. Epigenetic alterations in CCA

Histone modifications. Histones are basic proteins that
complex with genomic DNA to form nucleosomes, the basic
units of the compacted structure of chromatin. Histones are
modified post-translationally by acetylation, methylation and
phosphorylation. Histone acetylation appears to be the major
regulator of histone function. Histones are acetylated on
lysine residues at their amino termini by histone acetyltrans-
ferases (HATs), and acetylated histones are deacetylated by
histone deacetylases (HDACs). The HDAC-mediated removal
of acetyl groups from lysine residues at the amino termini of
histones leads to chromatin condensation and transcriptional
inactivation of the involved DNA (12,13). This transcriptional
inactivation can contribute to suppression of TSG expression
and enhanced tumorigenesis (14). In fact, HDAC inhibitors
enhance the acetylation state of histones, leading to chromatin
decondensation and increased gene expression (15,16).
HDAC inhibitors can, therefore, reverse the aberrant epigenic
state associated with cancer, and they have been shown to act
in synergy with DNA methylation inhibitors to inhibit tumor
growth (15,16). Nevertheless, there is only nominal
information on the etiologic roles of histone modifications in
cholangiocarcinogenesis.

DNA methylation. DNA methylation refers to the addition of
a methyl group to one of the four bases that constitute the
coding sequence of DNA (17,18). DNA methylation plays a
key role in chromatin structure, suppression of the activity of
endogenous parasitic sequences, and stable suppression of
gene expression (epigenetic silencing), a process normally
reserved for special situations, such as the inactive X-
chromosome and imprinted genes (19,20). DNA methyla-
tion occurs via the covalent addition of a methyl group to
the 5-position of the cytosine ring within the context of a
cytosine nucleotide followed by a guanine nucleotide (CpG
dinucleotide or CpG site) (18). It has been estimated that
almost half of the human gene promoter regions contain
CpG-rich regions, called CpG islands (21,22). Promoter
hypermethylation of CpG islands results in downregulation
or silencing of gene transcription (Fig. 1); thus, the aberrant
promoter hypermethylation of TSGs is an alternative
 mechanism of gene inactivation that contributes to the
carcinogenesis of human neoplasms, including CCA (4,6,22).

Genes hypermethylated in CCA. Genes hypermethylated in
the promoter CpG islands in CCA are summarized in Table I.
Through epigenetic silencing, TSGs are involved in important
molecular pathways of cholangiocarcinogenesis, e.g., cell-
cycle regulation, apoptosis, DNA repair and cell adhesion
(23), and are herein briefly reviewed.

p16⁰R⁰K⁰A, also called cyclin-dependent kinase inhibitor
2A, is a TSG located at human chromosome 9p21 that
inhibits interaction with cyclin D1 (24). This TSG is frequently
inactivated in a variety of tumors by deletion, mutations and
promoter hypermethylation (6). In CCA, CpG island methyla-
tion appears to be the main cause of p16⁰R⁰K⁰A inactivation
despite variable methylation frequencies (25-32). This
promoter hypermethylation has been shown to be
associated with a poor clinical outcome. The DNA methylation
frequencies of other genes related to cell cycle regulation,
including p1⁰A⁰R⁰F⁰⁰⁰R⁰F⁰³-⁰³, p1⁰⁵⁰I⁰N⁰K⁰⁴, p73⁰⁸, and ras association
domain family 1A (RASSF1A) (26,28,33,34), and their
chromosomal locations are shown in Table I.

Death-associated protein kinase (DAPK) gene is located
at chromosome 9q34.1, and its product is a proapoptotic
mediator of interferon-γ-induced programmed cell death
(4,35). The DNA methylation frequencies ranged from 3 to
32% in CCA (26,28,30). This promoter hypermethylation is
likely to be associated with poorly differentiated CCA and
with a poor prognosis (26,28). The DNA methylation
frequencies of other genes related to apoptosis, including
target of methylation-mediated silencing/apoptosis speck
like protein containing a caspase recruit domain (TMS1/
ASC) (36), semaphorin 3B (SEMA3B) (37), 14-3-3 (25), Runt-
related transcription factor 3 (RUNX3) (26), and checkpoint
with forkhead and ring finger domains (CHFR) (26), and
their chromosomal locations are shown in Table I.

Human mutL homologue 1 (hMLH1) is a DNA mismatch
repair gene located at 3p21.3 (38). Genetic and epigenetic
alterations of hMLH1 have been reported in various cancers
(38,39). In CCA, DNA methylation frequencies of the
hMLH1 gene promoter varied from 0 to 46% (26,28,30,40,41).
Limpaiboon et al found an association between this promoter
hypermethylation and poorly differentiated CCA with
vascular invasion (41). O⁰-methylguanine-DNA methyl-
transferase (MGMT) gene is located at chromosome 10q26.
This gene is responsible for repairing alkylated DNA
damage (42). Koga et al found a high methylation frequency
of 49% in MGMT gene (30), whereas Yang et al reported a
33% methylation frequency of MGMT gene in CCA (28).

Figure 1. (A) When CpG sites in the gene promoter are unmethylated, the
gene is transcribed to messenger RNA in most normal steady cells. (B) However, CpG island hypermethylation of the gene promoter inhibits
transcription factor-DNA interactions, leading to inhibition of gene expression.
This promoter hypermethylation can be associated with an increased frequency of GC to AT transitions in oncogenes and TSGs and with a poor prognosis (30).

Epithelial (E) cadherin gene is located at chromosome 16q22.1. The encoded protein is a calcium-dependent cell adhesion molecule (43). Genetic and epigenetic alterations in this gene lead to loss of function, permitting progression of cancer by increasing proliferation, invasion and metastasis (4,43). DNA methylation frequencies of this gene in CCA range from 22 to 43% (25,26,28,30).

Adenomatous polyposis coli (APC) gene is located at 5q21-q22. APC is a TSG that controls cell division, cell-cell interactions, and cell migration and invasion (4). APC gene hypermethylation ranges from 27 to 46% in CCA (25,28). This gene hypermethylation is associated with a worse clinical outcome in CCA patients (25).

The DNA methylation frequencies of retinoic acid receptor-ß 2 (RARß2; function, cell growth and differentiation) (28), glutathione S-transferase π 1 (GSTP1; function, drug/xenobiotic metabolism) (28), fragile histone triad (FHIT; function, purine metabolism) (33), Blunt protein (BLU; function, unknown) (37), methylated in tumor (MINT) 1, 12, 25, 31, 32 (function, unknown) (25), and their chromosomal locations are shown in Table I.

Suppressor of the cytokine signaling 3 (SOCS3) gene is at chromosome 17q25.3. The expression of this gene is induced by various cytokines, including interleukin-6 (IL-6), IL-10, and IFN-γ (44). SOCS3 can bind to and inhibit the activity of janus kinases (JAKs), turning off signal transducers and activators of transcription 3 (STAT3) signaling in response to IL-6 (Fig. 2) (44). Sustained overexpression of IL-6 has an integral role in CCA biology (45). Indeed, we demonstrated that IL-6-mediated STAT3 signaling is sustained in human CCA cells (46). More recently, we have shown that IL-6-mediated sustained STAT3 activation in human CCA is likely due to SOCS3 epigenetic silencing via hypermethylation of CpG islands within its promoter region (47), contributing to sustained IL-6/JAKs/STAT3 signaling in CCA cells. Thus, the loss of this negative regulator of IL-6 in CCA may contribute to cholangiocarcinogenesis.

Again, the downstream consequences of aberrant IL-6 expression may be further hypermethylation of the promoter regions of target genes in CCA (48). IL-6 has been shown to

| Table I. Promoter hypermethylation frequencies in various genes in cholangiocarcinoma. |
| Gene | Location | Function | Incidence (%) | Refs. |
| p14ARF | 9p21 | Cell cycle regulation | 25-38 | (28,32) |
| p15INK4b | 9p21 | Cell cycle regulation | 50 | (28) |
| p16INK4a | 9p21 | Cell cycle regulation | 18-83 | (25,26,27,28, 29,30,31,32) |
| p73 | 1p36.3 | Cell cycle regulation | 36-49 | (28) |
| RASSF1A | 3p21.3 | Cell cycle regulation | 27-69 | (26,28,33,34) |
| DAPK | 9q34.1 | Apoptosis | 3-32 | (26,28,30) |
| TMS1/ASC | 16p11.2 | Apoptosis | 5 | (36) |
| SEMA3B | 3p21.3 | Apoptosis | 100 | (37) |
| 14X3X3 | 1p36.11 | Apoptosis | 60 | (25) |
| RUNX3 | 1p36 | Apoptosis | 57 | (26) |
| CHFR | 12q24.33 | Apoptosis | 16 | (26) |
| hMLH1 | 3p21.3 | DNA repair | 8-46 | (26,28,30,40,41) |
| MGMT | 10q26 | DNA repair | 33-49 | (28,30) |
| RARß2 | 3p24.2 | Cell growth and differentiation | 14 | (28) |
| APC | 5q21 | Cell adhesion | 27-46 | (25,28) |
| E-cadherin | 16q22.1 | Cell adhesion | 22-43 | (25,26,28,30) |
| TIMP3 | 22q12.1 | Cell adhesion | 9 | (25,28) |
| THBS1 | 1q5 | Cell adhesion | 11 | (25,37) |
| GSTP1 | 11q13 | Drug/xenobiotic metabolism | 14 | (28) |
| FHIT | 3q14.2 | Purine metabolism | 42 | (33) |
| SOCS3 | 17q25.3 | Cytokine signaling | 88 | (45) |
| MINT1 | 22q11 | Unknown | 41 | (25) |
| MINT12 | 22q11 | Unknown | 51 | (25) |
| MINT25 | 22q11 | Unknown | 15 | (25) |
| MINT31 | 22q11 | Unknown | 1 | (25) |
| MINT32 | 22q11 | Unknown | 35 | (25) |
| BLU | 3p21.3 | Unknown | 20 | (37) |
regulate the enzyme activity of DNA methyltransferase responsible for DNA hypermethylation (49). For instance, the promoter methylation of epidermal growth factor receptor (EGFR) gene was decreased, and, consequently, EGFR gene and protein expressions were increased by IL-6 (48), suggesting epigenetic regulation of EGFR by the inflated IL-6 expression seen in CCA.

**Mir and CCA.** Mir is encoded in the genome of many species from plants to animals and is transcribed by RNA polymerase II as long precursor transcripts, which are known as primary mir (pri-mir) (50). Mature mir is generated from pri-mir by processing steps. The pri-mir is initially recognized by the microprocessor complex in the nucleus, whose core component is Drosha. The microprocessor complex excises the stem-loop hairpin structure that contains the pri-mir, a 60-80 nucleotide intermediate termed precursor mir (pre-mir). The pre-mir is recognized by exportin 5, which transports it to the cytoplasm (51,52). Pre-mir is exported to the cytoplasm by exportin 5. Further cytoplasmic processing by Dicer performs a second cleavage to generate a double-stranded 18-24 nucleotide-long mir duplex. One of these two strands is incorporated into RNA-induced silencing complex, RISC. Only one strand of the mir duplex remains stably associated with RISC. This strand becomes the mature mir. The mature mir guides the RISC complex to the target mRNA, which is then cleaved or translationally silenced.

**Figure 2. Interleukin 6 (IL-6) binds to its cognate receptor (IL-6R), leading to activation of janus kinases (JAKs). Following phosphorylation (activation) of signal transducers and activators of transcription 3 (STAT3) by JAKs, it dimerizes and translocates into the nucleus, where the STAT3 dimer binds to the promoter region of suppressor of the cytokine signaling 3 (SOCS3) gene. SOCS3 can bind to and inhibit the activity of Janus kinases (JAKs), turning off STAT3 signaling in response to IL-6.**

**Figure 3. MicroRNA (mir) is encoded in the genome and is transcribed by RNA polymerase II as long precursor transcripts, which are known as primary miRNA (pri-mir). The pri-mir is initially recognized by the microprocessor complex in the nucleus, whose core component is Drosha. The microprocessor complex excises the stem-loop hairpin structure that contains the pri-mir, a 60-80 nucleotide intermediate termed precursor mir (pre-mir). The pre-mir is recognized by exportin 5, which transports it to the cytoplasm. Further cytoplasmic processing by Dicer performs a second cleavage to generate a double-stranded 18-24 nucleotide-long mir duplex. One of these two strands is incorporated into RNA-induced silencing complex, RISC. Only one strand of the mir duplex remains stably associated with RISC. This strand becomes the mature mir. The mature mir guides the RISC complex to the target mRNA, which is then cleaved or translationally silenced.**

To date, the data concerning mir regulation of cholangiocarcinogenesis is sparse. Meng et al showed that mir-141 was overexpressed in human CCA cells (60). Inhibiting mir-141 effectively increased the expression of CLOCK, which regulates circadian rhythms and can act as a tumor suppressor in CCA. They also found overexpression of mir-200b and its target, the protein tyrosine phosphatase non-receptor type 12, the dysregulation of which may contribute to tumor cell survival and carcinogenesis. Similarly, the expression of mir-21 was overexpressed in CCA, which blocks tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome 10) expression (61).

Again, enforced IL-6 overexpression in human CCA cell lines significantly increased let-7a expression (62). Let-7a is likely to contribute to survival effects attributable to the inflated IL-6. A putative target of let-7a is the neurofibromatosis 2 (NF2) gene (62), which is a negative regulator of STAT3 (63). Thus, overexpression of IL-6 in CCA and subsequent up-regulation of let-7a decrease NF2 expression, thereby removing the negative regulation of STAT3, which may be linked to constitutive activation of STAT3, the pivotal transcription factor implicated in a number of cancers, including CCA.

Conversely, other mir species have been identified as being downregulated in CCA cells compared to non-malignant
cholangiocytes. In terms of carcinogenesis, these types of mir are considered as TSG. Mir-29b expression was suppressed in a CCA cell line, and enforced mir-R-29b expression effectively reduced the target molecule, myeloid cell leukemia 1, an anti-apoptotic protein of the Bcl-2 family (64), possibly leading to apoptotic resistance of CCA. Interestingly, mir-370, whose expression was substantially reduced in CCA cells, was shown to be under epigenetic regulation by DNA hypermethylation (65).

Other epigenetic mechanisms and CCA. More recently, Sasaki et al. revealed that p16INK4a promoter hypermethylation was related to aberrant expression of enhancer of zeste Drosophila homologue 2 (EZH2), a component of the polycomb family of gene-silencing proteins, in cholangiocarcinogenesis in hepatolithiasis (66). There is a mechanism in which modifications of histones mark a gene for hypermethylation by the binding of methyltransferase enhancers such as EZH2 to histones (67).

4. Conclusions

DNA methylation and histone modifications are the hallmarks of epigenetic gene regulation. Mir is a recently discovered category of non-coding RNAs with important regulatory functions. Aberrances in both the epigenetic and the miRNA regulation of genes have been documented in CCA. A deeper understanding of the association of epigenetic mechanisms with CCA could lead to new therapeutic avenues.

References