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## Genetic and epigenetic changes associated with cholangiocarcinoma: From DNA methylation to microRNAs

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### Abstract

Cholangiocarcinomas are malignant epithelial liver tumors arising from the intra- and extra-hepatic bile ducts. Little is known about the molecular development of this disease, and very few effective treatment options are available. Thus, prognosis is poor. Genetic and epigenetic changes play an integral role in the neoplastic transformation of human cells to their malignant counterparts. This review summarizes some of the more prevalent genetic alterations (by microRNA expression) and epigenetic changes (hypermethylation of specific gene promoters) that are thought to contribute to the carcinogenic process in cholangiocarcinoma.

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### INTRODUCTION

Neoplastic transformation of normal cells into their malignant counterparts often requires a series of genetic

changes. These changes can range from "simple" mutations in the genes themselves that ultimately lead to loss-of-function or gain-of-function changes in key genes that are responsible for the control of apoptosis and cell cycle progression respectively, to more complex changes in non-protein factors (e.g. RNA and DNA) that regulate the control of specific gene expression<sup>[1-4]</sup>. These more complex changes can be classified as either genetic or epigenetic changes<sup>[1,3,4]</sup>. For the purpose of this review, we will define and limit the "complex" genetic changes as being any changes that occur in non-coding RNAs and epigenetic changes in the modification of the promoter regions of the genes themselves (e.g. hyper- and hypomethylation)<sup>[3-6]</sup>.

### Non-coding RNAs

Non-coding RNAs have recently been recognized as gene-specific regulators and therefore are similar in function to transcription factors. These RNAs can regulate every stage of gene expression, including transcription, mRNA stability and translation<sup>[5,7]</sup>. A role for these RNAs in the neoplastic transformation of cells is emerging<sup>[4]</sup>. A major sub-type of non-coding RNA is the microRNAs, which are small RNA molecules encoded in the genomes of plants and animals<sup>[5]</sup>. These highly conserved, about 21-mer RNAs regulate the expression of specific target genes by binding to the 3'-untranslated regions of mRNAs<sup>[7]</sup>. Each microRNA is thought to regulate multiple genes and since hundreds of microRNA molecules are predicted<sup>[6]</sup>, the relative importance of these molecules in the control of gene expression is potentially massive. Several research groups have provided evidence that microRNAs may act as key regulators of processes as diverse as early development<sup>[8]</sup>, cell proliferation and cell death<sup>[9]</sup>, apoptosis and fat metabolism<sup>[10]</sup>, and cell differentiation<sup>[11,12]</sup>. In addition, recent studies suggest a possible link between microRNAs and various cancers, including chronic lymphocytic leukemia<sup>[13]</sup>, colonic adenocarcinoma<sup>[14]</sup>, Burkitt's Lymphoma<sup>[15]</sup> and cholangiocarcinoma<sup>[16-18]</sup>.

### Epigenetic changes

Epigenetic gene silencing refers to non-mutational gene inactivation that can be passed from parental cells to daughter cells<sup>[1]</sup>. The addition of methyl groups to cytosine residues in CpG dinucleotides in DNA is a biochemical modification that meets this requirement, referred to as hypermethylation<sup>[1]</sup>. Genes carrying epimutations

cause morphological phenotypes to be transmitted from generation to generation, not based on any alteration in the coding sequence of the relevant genes, but instead caused by CpG (or CpNpG) hypermethylation of their promoter sequences<sup>[2]</sup>. Methylation patterns in mammalian cells are regulated by DNA methyltransferases<sup>[19]</sup>, which transfer a methyl group to the cytosine portion of the CpG dinucleotide<sup>[19]</sup>. This allows for the binding of methyl-specific DNA-binding proteins such as MeCP1 or MeCP2 to regulatory elements, which in turn represses transcription<sup>[20]</sup>. These binding proteins can then attract histone deacetylases, which then remodel chromatin into highly repressed states<sup>[20]</sup>. Hypermethylation has been shown to play an important role in the progression of tumor growth in almost all types of cancer<sup>[21]</sup>.

Recent data suggest that both genetic and epigenetic changes are required for transformation, promotion and progression of cholangiocarcinoma<sup>[16,17,22-26]</sup>, a deadly cancer of the cells lining the intra- and extra-hepatic biliary tract<sup>[27,28]</sup>. This type of cancer is increasing in its incidence worldwide, with no effective treatment options<sup>[29,30]</sup>. Therefore, understanding the molecular events associated with the neoplastic transformation of cholangiocytes to cholangiocarcinoma may aid in the development of improved therapeutic strategies. This review will summarize our current understanding of the most prevalent genetic and epigenetic changes associated with cholangiocarcinoma.

## THE REGULATION OF TUMOR SUPPRESSOR GENES AND CELL CYCLE INHIBITORS

### Methylation

The hypermethylation and inactivation of a number of cell cycle inhibitors have been shown to occur in cholangiocarcinomas. The most well-characterized of these epigenetic changes is in the p16<sup>INK4a</sup> gene, which has been described in up to 83% of cholangiocarcinomas<sup>[23,26]</sup>. This gene is responsible for binding to the cyclin dependent kinase 4 (CDK4) and inhibits its ability to interact with cyclin D1<sup>[31]</sup>. In the absence of p16<sup>INK4a</sup> activity, such as after promoter methylation, CDK4 binds to cyclin D1, which subsequently leads to unchecked entry into the S phase of the cell cycle<sup>[31]</sup>. There also appears to be increased incidence of hypermethylation of the related p14<sup>ARF</sup> occurring in 25% of cholangiocarcinoma samples studied<sup>[25]</sup>. The p14<sup>ARF</sup> gene normally prevents p53 degradation and hence cell-cycle arrest<sup>[32]</sup>, which constitutes another checkpoint that may be lost in cholangiocarcinoma<sup>[33]</sup>. In addition to the above-mentioned genes, many other cell cycle entry inhibitors have been shown to be hypermethylated. These include p16<sup>INK4b</sup> (50% of tumors studied)<sup>[26]</sup> and 14-3-3 sigma (59.5% of tumors studied)<sup>[34]</sup>.

In addition, the expression of many tumor suppressor genes is repressed in cholangiocarcinomas<sup>[24]</sup>. The most striking of which is Semaphorin3B, which was found to be methylated in 100% of the cholangiocarcinoma cases studied<sup>[24]</sup>. RassF1A<sup>[26]</sup> and p73<sup>[26]</sup> are also

hypermethylated and suppressed in 65.3% and 36.1% of cholangiocarcinoma cases studied respectively.

Taken together, the epigenetic silencing of a vast array of tumor suppressor genes and inhibitors of cell cycle progression obviously plays an important role in the initiation and progression of cholangiocarcinoma. Inactivation of these genes allows cells to avoid apoptosis and to proliferate unchecked.

### microRNA

To date, the data concerning microRNA regulation of cell cycle and/or apoptotic genes is sparse. Recently Meng *et al* showed that miR-141 was highly overexpressed in malignant cholangiocytes<sup>[16]</sup>. Using a bioinformatics approach, a predicted target of miR-141 was the CLOCK gene, which regulates circadian rhythms and can act as a tumor suppressor<sup>[16]</sup>. Inhibiting miR-141 effectively increased CLOCK protein expression in cholangiocarcinoma cells<sup>[16]</sup>. Another microRNA species that was found to be overexpressed in malignant cholangiocytes was miR-200b<sup>[16]</sup>. The target gene for this was predicted to be the protein tyrosine phosphatase non-receptor type 12 (PTPN12), the dysregulation of which may contribute to tumor cell survival and oncogenesis<sup>[16]</sup>. Similarly, the expression of miR-21 was overexpressed in cholangiocarcinoma, which effectively blocks the expression of the tumor suppressor gene PTEN<sup>[16]</sup>.

Conversely, other microRNA species have been identified as being downregulated in cholangiocarcinoma compared to non-malignant cholangiocytes. miR-29b expression was suppressed in the cholangiocarcinoma cell line KMCH as well as in approximately 33% of human cholangiocarcinoma samples<sup>[18]</sup>. Enforced miR-29b overexpression in cholangiocarcinoma cells effectively reduced the expression of Mcl-1, an anti-apoptotic protein of the Bcl-2 family<sup>[18]</sup> and sensitized cholangiocarcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand cytotoxicity, suggesting that the suppression of miR-29b expression found in cholangiocarcinoma allows the overexpression of Mcl-1 and can ultimately lead to the resistance of cholangiocarcinoma to cell death<sup>[18]</sup>. Another microRNA that is downregulated in cholangiocarcinoma is miR-370<sup>[17]</sup>. Interestingly, the expression of this particular microRNA has been shown to be under tight epigenetic regulation by hypermethylation<sup>[17]</sup>. One of the targets for miR-370 is the oncogene mitogen-activated protein kinase kinase kinase 8 (MAP3K8)<sup>[17]</sup>, thus MAP3K8 is upregulated in cholangiocarcinoma cell lines as well as in tumor cell xenografts *in vivo*<sup>[17]</sup>.

## THE REGULATION OF GENES INVOLVED IN DNA REPAIR

### Methylation

Inherited mutations that affect DNA repair genes are strongly associated with high cancer risks in humans. A number of the DNA repair genes have been shown to be hypermethylated in cholangiocarcinoma. Hypermethylation of the hMLH1 mismatch repair gene promoter has been shown to occur in up to 23.6%

of cholangiocarcinomas<sup>[26]</sup>, which has previously been revealed to lead to microsatellite instability in other tumor types<sup>[35]</sup>. Another gene, O6-methylguanine-DNA methyltransferase (MGMT), is silenced in up to 33% of cholangiocarcinoma tumors studied<sup>[26]</sup>. This gene is an important suicide enzyme involved in the defense against O6-alkylating endogenous metabolites and environmental carcinogens. Interestingly, transcriptional repression of MGMT was associated with the accumulation of GC-AT transitional mutations in the p53 gene and, to a lesser extent, the *k-ras* gene in cholangiocarcinoma<sup>[26,36]</sup>. Lastly, the glutathione S-transferase P1 (GSTP1) gene, which inactivates electrophilic carcinogens by conjugation with glutathione is hypermethylated in cholangiocarcinoma, occurring in 6% to 34% of cases studied<sup>[26,34]</sup>. Taken together, the hypermethylation of genes responsible for DNA repair appears to be an important step in the carcinogenic process of cholangiocarcinoma.

### microRNA

To our knowledge, and to date, no genes involved in DNA repair have been identified as targets of transcriptional control by microRNA in cholangiocarcinoma.

## THE REGULATION OF GENES INVOLVED IN INFLAMMATION

### Methylation

Chronic inflammation, such as those seen in various cholestatic liver diseases, is a recognized risk factor for the development of cholangiocarcinoma<sup>[27,28]</sup>. Therefore, it follows that certain mediators of the inflammatory process may be integral in the carcinogenic processes associated with neoplastic transformation of cholangiocytes<sup>[37-39]</sup>. Indeed, sustained overexpression of the cytokine interleukin-6 (IL-6) has been demonstrated to have an integral role in cholangiocarcinoma biology<sup>[38,39]</sup>. This aberrant overexpression of IL-6 has recently been shown to be as a consequence of the epigenetic silencing of the suppressor of cytokine signaling 3 (SOCS-3)<sup>[40]</sup>. SOCS-3 promoter methylation was observed in a subset of cholangiocarcinoma samples as well as in a number of cholangiocarcinoma cell lines<sup>[40]</sup>. Enforced overexpression of SOCS-3 in these cell lines effectively reduced the IL-6-mediated signal transduction cascade<sup>[40]</sup>. Therefore the loss of this negative regulator of IL-6 in cholangiocarcinoma may contribute to the inflated expression and activity of inflammatory molecules seen in cholangiocarcinoma.

A downstream consequence of aberrant IL-6 expression may be the further hypermethylation of the promoter regions of a number of critical target genes in cholangiocarcinoma. IL-6 has been shown to regulate the enzyme activity of one of the DNA methyltransferases responsible for the hypermethylation of promoter regions<sup>[41]</sup>. In cholangiocarcinoma cells, IL-6 overexpression resulted in the altered promoter methylation of a number of genes including the epidermal growth factor receptor (EGFR)<sup>[42]</sup>. EGFR promoter methylation was decreased and gene and protein expression increased by IL-6<sup>[42]</sup>, suggesting that the

epigenetic regulation of gene expression by the inflated IL-6 expression seen in cholangiocarcinoma can contribute to tumor progression by altering the expression of growth regulatory pathways, such as those involving EGFR<sup>[42]</sup>.

### microRNA

In addition to changes in promoter methylation, aberrant IL-6 expression in cholangiocarcinoma also has implications on microRNA expression and function. Enforced IL-6 overexpression in human cholangiocarcinoma cell lines significantly increased the expression of several members of the let-7 family of microRNAs<sup>[22]</sup>. Expression of let-7a contributes to the survival effects that are attributed to IL-6 overexpression<sup>[22]</sup>. A putative target of let-7a microRNA is the gene neurofibromatosis 2 (NF2)<sup>[22]</sup>, which is a negative regulator of Stat-3<sup>[43]</sup>. Thus, overexpression of IL-6 in cholangiocarcinoma and subsequent upregulation of let-7a decreased the expression of NF2, thereby removing the negative regulation of Stat-3<sup>[22]</sup>. Constitutive activation of Stat-3 has been implicated in a number of cancers and is thought to be responsible for the IL-6-mediated survival signaling<sup>[22]</sup>.

## THE REGULATION OF GENES INVOLVED IN CELL ADHESION AND INVASION

### Methylation

E-Cadherin is a calcium-dependent cell adhesion molecule that suppresses metastatic processes and tumor cell invasion<sup>[44-46]</sup>. In cholangiocarcinoma, methylation of the E-cadherin promoter occurred in up to 48% of all samples studied<sup>[25,26,34,47]</sup>. Downregulation of this gene by epigenetic changes has been reported in a number of other cancers<sup>[44,45]</sup>, and re-expression can be induced by treatment with a demethylating agent in cholangiocarcinoma<sup>[48]</sup>.

### microRNA

To our knowledge, no genes that are involved in cell adhesion or the metastatic process associated with cholangiocarcinoma biology have been shown to be regulated by microRNAs.

## CONCLUSION

In conclusion, from the epigenetic changes summarized in this review, it is obvious that epigenetic silencing as well as genetic regulation by microRNAs play a very important role in the neoplastic transformation of cholangiocytes to their malignant counterparts. A summary of the changes in specific promoter hypermethylation and microRNA can be found in Tables 1 and 2 respectively. We acknowledge that this summary is far from complete and that more target genes are being described regularly. Further analysis and characterization of these genetic and epigenetic changes, as well as the potential interplay between hypermethylation and microRNA expression will aid in the identification of therapeutic targets for the design of treatment strategies to combat this deadly malignancy.



**Table 1** Summary of genes subject to epigenetic silencing during the course of cholangiocarcinoma tumor progression

Gene	Incidence of methylation in CCH (%)	Function	Reference
p16 <sup>INK4a</sup>	14-50	Cell cycle regulator	[26,34,47]
p14 <sup>ARF</sup>	38	Cell cycle regulator	[26]
p15 <sup>INK4b</sup>	12-50	Cell cycle regulator	[26,47]
14-3-3 sigma	59.50	Cell cycle regulator	[34]
Semaphorin3B	100	Tumor suppressor	[24]
p73	36	Tumor suppressor	[26]
RassF1A	26-65	Tumor suppressor	[26,47]
hMLH1	25	DNA mismatch repair	[26]
MGMT	11-33	Methyl transferase	[26,34]
GSTP1	6-34	Inactivation of carcinogens	[26,34]
SOCS-3	ND	Inhibits inflammation	[40]
EGFR	ND	Growth factor	[42]
E-cadherin	43	Cell adhesion	[25,26,34,47]

ND: Not determined.

**Table 2** MicroRNAs known to be changed in cholangiocarcinoma

MicroRNA	Target gene	Function	Direction changed in CCH	Reference
miR-141	CLOCK	Circadian rhythm	Increased	[16]
miR-200b	PTPN12	Tumor suppressor	Increased	[16]
miR-21	PTEN	Tumor suppressor	Increased	[16]
miR-29b	Mcl-1	Anti-apoptotic gene	Decreased	[18]
miR-370	MAP3K8	Oncogene	Decreased	[17]
let-7a	NF2	Negative regulator of inflammation	Increased	[22]

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