Epigenetic Alterations in Cholangiocarcinoma- Sustained IL-6/STAT3 Signaling in Cholangio- carcinoma due to SOCS3 Epigenetic Silencing

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Key Words

Cholangiocarcinoma • Epigenetics • DNA hypermethylation • Carcinogenesis • Suppressor of cytokine signaling 3 • Interleukin-6 • Signal transducers and activators of transcription 3

Abstract

Cholangiocarcinoma (CCA) is a highly lethal malignant tumor arising from the biliary tract epithelium, characterized by its typically late clinical presentation and lack of effective therapeutic modalities. Chronic inflammatory conditions, including primary sclerosing cholangitis, liver fluke infestation and hepatolithiasis, are listed in the risk factors, but for most cases of CCA the cause is unknown. Recent advances in molecular pathogenesis have highlighted the importance of epigenetic alterations including promoter hypermethylation and histone deacetylation in addition to genetic changes in the process of cholangiocarcinogenesis. This review provides a comprehensive overview of the genes hypermethylated in CCA to date and their putative roles in cholangiocarcinogenesis. Among genes hypermethylated, we found the CpG island hypermethylation in suppressor of cytokine signaling 3 (SOCS3) gene promoter in CCA. Interleukin-6 (IL-6)-mediated signal transducers and activators of transcription 3 (STAT3) activation are aberrantly sustained in CCA cells, resulting in resistance to apoptosis. SOCS3 controls the IL-6/STAT3 signaling pathway by a classic feedback loop. Indeed, SOCS3 epigenetic silencing is responsible for

sustained IL-6/STAT3 signaling in CCA. These findings provide new perspectives for epigenetic therapy to restore SOCS3 in this cancer.

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Introduction

Cholangiocarcinoma (CCA) is a devastating neoplasm originating from cholangiocytes, the epithelial cells that line the biliary apparatus [1]. It comprises only 10–15% of hepatobiliary neoplasm [2, 3]. Two thirds of CCA involve the extrahepatic bile duct, whereas the remaining 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 [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 molecular mechanisms in CCA. As for many other tumors, the development of CCA must also 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.

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 stresses and irreversible DNA damage [1, 2]. As a result, cholangiocytes attain cellular phenotypes that result in malignant transformation [1]. The proposed pathways that partake in cholangiocarcinogenesis include: self-sufficiency and proliferation; apoptosis resistance; escape from senescence and tumor invasiveness and metastasis [1]. Molecular mechanisms responsible for the 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 of APC; 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 owing to differences in the subsites of cancers, racial and geographical variations in the study populations and the use of different assay techniques [4].

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 posttranslationally by acetylation, methylation and phosphorylation. Histone acetylation appears to be the major means of regulation of histone function. Histones are acetylated on lysine residues at their amino termini by histone acetyltransferases and acetylated histones are deacetylated by histone deacetylases (HDACs). The HDAC-mediated removal of acetyl groups from lysine

residues in 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 epigenetic state associated with cancer and have been shown to act in synergy with DNA methylation inhibitors to inhibit tumor growth [15, 16]. There is, nevertheless, nominal information on the etiologic roles of histone modifications in cholangiocarcinogenesis.

DNA Methylation

DNA methylation refers to the addition of a methyl group to 1 of the 4 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 methylation 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 the CpG-rich regions, called CpG islands [21, 22]. Promoter hypermethylation of CpG islands results in downregulation or silencing of gene transcription, and 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 1. TSGs through epigenetic silencing are involved in important molecular pathways of cholangiocarcinogenesis, such as cell-cycle regulation, apoptosis, DNA repair and cell adhesion [23], and are herein reviewed in brief.

p16^{INK4A}, also called cyclin-dependent kinase inhibitor 2A, is a TSG located at human chromosome 9p21 and inhibits their 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 methylation appeared to be the main cause of p16^{INK4a} inactivation despite variable methylation fre-

Table 1. Promoter hypermethylation frequencies in various genes in cholangiocarcinoma

Gene	Location	Function	Incidence, %	Reference
p14 ^{ARF}	9p21	cell cycle regulation	25–38	28, 32
p15 ^{INK4b}	9p21	cell cycle regulation	50	28
p16 ^{INK4a}	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
RAR β 2	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	15q15	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

quencies [25–32]. This promoter region 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 p14^{ARF} [28, 32], p15^{INK4b} [28], p73 [28] and Ras association domain family 1A (RASSF1A) [26, 28, 33, 34], as well as their chromosomal locations are shown in table 1.

Death-associated protein kinase (DAPK) gene is located at chromosome 9q34.1 and its product is a proapoptotic mediator of IFN- 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 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], as well as their chromosomal locations are shown in table 1.

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 0–46% [26, 28, 30, 40, 41]. Limpaboon et al. [41] found an association between this promoter hypermethylation and the poorly differentiated CCA with vascular invasion. O⁶-methylguanine-DNA methyltransferase (MGMT) gene is located at chromosome 10q26. This gene is responsible for repairing alkylation DNA damage [42]. Koga et al. [30] found a high methylation frequency of 49% in MGMT gene, whereas Yang et al. [28] reported a 33% methylation frequency of MGMT gene in CCA. 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 cadherin (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 ranged 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 ranged 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 other genes related to cell adhesion, including tissue inhibitor of metalloproteinase (TIMP3) [25, 28] and thrombospondin 1 (THBS1) [25, 37], as well as their chromosomal locations are shown in table 1.

The DNA methylation frequencies including retinoic acid receptor- β 2 (RAR β 2; function: cell growth and differentiation) [28], glutathione S-transferase pi 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; function: unknown) [1, 12, 25, 31, 32] as well as their chromosomal locations are shown in table 1.

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 (IL)-6, IL-10 and IFN- γ [44]. The protein encoded by this gene can bind to and inhibit the activity of Janus kinases, turning off signal transducers and activators of transcription 3 (STAT3) signaling in response to IL-6 [44]. 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 (fig. 1) [45], and described the outlines below.

Sustained IL-6/STAT3 Signaling in CCA due to SOCS3 Epigenetic Silencing

We have previously demonstrated that IL-6-mediated STAT3 signaling is sustained in human CCA cells [46]. Following binding by IL-6 to its cognate receptor complex, Janus kinases are recruited to the receptor complex and activated [44, 47]. These kinases phosphorylate STAT3 which then dimerizes and translocates to the nucleus where it functions as a transcription factor [44, 47]. Normally, the STAT3 response to IL-6 stimulation is ter-

Fig. 1. CpG island hypermethylation of SOCS3 gene promoter inhibits transcription factor-DNA interactions, leading to inhibition of SOCS3 expression.

minated by SOCS3, a negative regulator of IL-6/STAT3 signaling [44]. In a classic feedback loop, STAT3 dimers turn on the SOCS3 gene which then feeds back and inhibits the signaling cascades [44, 47].

We generated the hypothesis that SOCS3 silencing results in sustained IL-6/STAT3 signaling in human CCA cells. Silencing of the SOCS3 gene would, indeed, explain why IL-6 signaling in these cells is not turned off as in normal cells. In the first place, we sought to investigate the relationship between activated STAT3 and SOCS3 expression in human CCA tissue by immunohistochemistry. We found an inverse relationship between activated STAT3 and SOCS3 cytoplasmic expression in human CCA tissue; STAT3 nuclear immunoreactivity was observed, whereas SOCS3 expression was absent (fig. 2).

Tissue-specific genomic DNA was isolated from the human CCA tissue sections by laser capture microdissection. The genomic DNA was subjected to methylation-specific PCR and then the bisulfite-modified DNA was sequenced. SOCS3 promoter was methylated in human CCA; the methylation-specific PCR showed intense methylation PCR product was observed in the SOCS3 promoter from CCA, while the SOCS3 promoter was unmethylated in paired nonmalignant tissue (uninvolved bile duct epithelial cells and hepatocytes) from the same specimen (fig. 3).

Next, we performed sequencing the bisulfite-modified genomic DNA to assess CpG island methylation directly. DNA enriched for CpG Islands were sequenced between regions -678 and -216 of the promoter. Five random clones were each sequenced. The SOCS3 promoter was densely methylated in the cancer but not in the unin-

Fig. 2. Inverse correlation between tyrosine⁷⁰⁵-phosphorylated STAT3 and SOCS3 expression in human CCA tissue. Arrows indicate intense nuclear localization of activated STAT3 and absent expression of cytoplasmic SOCS3 in the serial sections.

Color version available online

Fig. 3. Methylation-specific PCR was performed using the methylated and unmethylated DNA-specific primer sets. CpG island methylation of the SOCS3 promoter is specific for CCA. Genomic DNA was extracted from paired tumor and nontumor tissue (uninvolved bile duct epithelial cells and hepatocytes) procured by laser capture microdissection.

Fig. 4. PCR products were sequenced for the 44 CpG sites located between nucleotides -678 and -216 of the SOCS3 promoter following bisulfate modification. The SOCS3 promoter was densely methylated in the cancer but not in the uninvolved tissue.

involved tissue (fig. 4); it would appear that CpG island methylation of the SOCS3 promoter is a potential mechanism for silencing of this gene in human CCA (fig. 1). To ascertain the functional significance of this methylation, we examined a CCA cell line retain the SOCS3 promoter methylation pattern. Employing the malignant Mz-ChA-1 cells and the nonmalignant H69 cells as a control, the methylation status of the SOCS3 promoter was determined by methylation-specific PCR and bisulfite sequencing. CpG island methylation of the SOCS3 promoter was observed in the malignant but not in the nonmalignant cells. Methylation-specific PCR demonstrates a methylated product for the SOCS3 promoter in the cancer cells but not in the nonmalignant cells. Likewise, dense methylation of the CpG Islands in the promoter

region was observed in the cancer cell line but not in the nonmalignant cells. Thus, this CCA cell line retains a similar pattern of SOCS3 promoter methylation as does the human tissue.

To determine whether epigenetic silencing of SOCS3 is responsible for the sustained STAT3 activation by IL-6, the MZ-ChA-1 cells were treated with a demethylating agent, 5-aza-2'-deoxycytidine (DAC) and then the expression of activated STAT3 and SOCS3 was assessed in response to IL-6 by real-time PCR and immunoblot analysis. SOCS3 was not expressed in these cells unless they were pretreated with DAC, but following exposure to the demethylating agent, IL-6 induced SOCS3 expression (fig. 5). Again, there was a prolonged phospho-STAT3 activation in response to IL-6 when treated with vehicle. In

Fig. 5. A CCA cell line; MZ-ChA-1 cells were treated with a demethylating agent, DAC, and then the expression of SOCS3 was assessed in response to IL-6 by real-time PCR and immunoblot analysis. DAC treatment restores SOCS3 expression in response to IL-6 in the cancer cells.

contrast, when the cells are pretreated with DAC, SOCS3 re-expression terminated the phospho-STAT3 signaling event. Thus, the promoter methylation appears to be functional in silencing the SOCS3 gene, and this is a reversible process.

In conclusion, the SOCS3 silencing contributes to the sustained IL-6/STAT3 signaling in CCA cells. The demethylating agents can reverse this process, suggesting that epigenetic therapy may be potentially useful in the treatment of CCA.

Disclosure Statement

The author declares that no financial or other conflict of interest exists in relation to the content of the article.

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