Omega-3 Polyunsaturated Fatty Acids Upregulate 15-PGDH Expression in Cholangiocarcinoma Cells by Inhibiting miR-26a/b Expression

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Abstract

Prostaglandin E₂ (PGE₂) is a proinflammatory lipid mediator that promotes cancer growth. The 15-hydroxyprostaglandin dehydrogenase (15-PGDH) catalyzes oxidation of the 15(S)-hydroxyl group of PGE₂, leading to its inactivation. Therefore, 15-PGDH induction may offer a strategy to treat cancers that are driven by PGE₂, such as human cholangiocarcinoma. Here, we report that omega-3 polyunsaturated fatty acids (ω-3 PUFA) upregulate 15-PGDH expression by inhibiting miR-26a and miR-26b, thereby contributing to ω-3 PUFA-induced inhibition of human cholangiocarcinoma cell growth. Treatment of human cholangiocarcinoma cells (CCLP1 and TKF-1) with ω-3 PUFA (DHA) or transfection of these cells with the Fat-1 gene (encoding Caenorhabditis elegans desaturase, which converts ω-6 PUFA to ω-3 PUFA) significantly increased 15-PGDH enzymes levels, but with little effect on the activity of the 15-PGDH gene promoter. Mechanistic investigations revealed that this increase in 15-PGDH levels in cells was mediated by a reduction in the expression of miR-26a and miR-26b, which target 15-PGDH mRNA and inhibit 15-PGDH translation. These findings were extended by the demonstration that overexpressing miR-26a or miR-26b decreased 15-PGDH protein levels, reversed ω-3 PUFA-induced accumulation of 15-PGDH protein, and prevented ω-3 PUFA-induced inhibition of cholangiocarcinoma cell growth. We further observed that ω-3 PUFA suppressed miR-26a and miR-26b by inhibiting c-myc, a transcription factor that regulates miR-26a/b. Accordingly, c-myc overexpression enhanced expression of miR-26a/b and ablated the ability of ω-3 PUFA to inhibit cell growth. Taken together, our results reveal a novel mechanism for ω-3 PUFA-induced expression of 15-PGDH in human cholangiocarcinoma and provide a preclinical rationale for the evaluation of ω-3 PUFA in treatment of this malignancy. Cancer Res; 75(7): 1388–98. © 2015 AACR.

Introduction

Cholangiocarcinoma is a highly malignant cancer of the biliary tract with poor prognosis (1–12). The incidence and mortality of cholangiocarcinoma is rising worldwide and currently there is no effective chemoprevention or treatment. The tumor often arises from background conditions that cause long-standing inflammation, injury, and reparative biliary epithelial cell proliferation, such as primary sclerosing cholangitis, clonorchiasis, hepatolithiasis, or complicated fibropolyposic diseases. Intrahepatic cholangiocellular carcinoma is also associated with hepatitis C viral infection and liver cirrhosis secondary to other nonbiliary cancers. The carcinogenic processes involve a series of sequential events, including chronic inflammation, cholangiocyte proliferation, dysplasia, and ultimately malignant transformation.

Consistent with the strong association between bile duct chronic inflammation and cholangiocarcinogenesis, compelling evidence (10, 13–28) has shown that mediators of inflammation, such as prostaglandins, play an important role in cholangiocarcinogenesis. For example, elevated expression of COX-2 has been documented in cholangiocarcinoma cells and precancerous bile duct lesions, but not in normal bile duct epithelial cells (21–25); the expression of COX-2 is induced by proinflammatory cytokines as well as bile acids (29, 30). Overexpression of COX-2 in human cholangiocarcinoma cells enhances prostaglandin E₂ (PGE₂) production and promotes tumor growth, whereas depletion of COX-2 reduces PGE₂ production and prevents growth (14, 15). PGE₂ treatment is known to increase cholangiocarcinoma cell growth and prevent apoptosis (13–15, 26–28). Accordingly, inhibition of COX-2 by molecular and pharmacologic approaches prevents the growth and invasion of cholangiocarcinoma cells in vitro and in animal models (10, 14, 15, 22, 25, 27, 28). These findings provide important preclinical evidence for targeting COX-2 in prevention and treatment of human cholangiocarcinoma. However, as some COX-2 inhibitors are known to be associated with increased cardiovascular side effect (31–34), there is an urgent and practical need to identify COX-2 downstream target for effective anti-cholangiocarcinoma therapy with fewer side effects.

The amount of biologically active PGE₂ in the inflammatory and tumor microenvironment is regulated by the balance between PGE₂ synthesis and degradation. Although previous studies have focused on the role of COX-2 in carcinogenesis, the role of PGE₂
degradation enzyme, the NAD⁺-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH), has not been recognized until recently. 15-PGDH catalyzes oxidation of the 15(S)-hydroxyl group of PGE₂, converting PGE₂ into 15-keto-PGE₂; this enzymatic reaction leads to reduction of the proinflammatory and protumorigenic PGE₂ (35). Indeed, accumulating evidence suggests that 15-PGDH is an important tumor suppressor in a number of human cancers, including cholangiocarcinoma (36).

Although the proinflammatory and procarcinogenic prostaglandins are synthesized from arachidonic acid (AA), a ω-6 PUFA; this process is competitively inhibited by ω-3 polyunsaturated fatty acids (ω-3 PUFAs). The lipid mediators derived from ω-6 and ω-3 PUFA are metabolically distinct and often have opposing physiologic and pathologic functions; for example, ω-6 PUFA-derived eicosanoids tend to promote inflammation and carcinogenesis, whereas ω-3 PUFA-derived lipid mediators largely inhibit inflammation and prevent carcinogenesis (or less promotional for inflammation and proliferation). In this study, we report that ω-3 PUFA (but not ω-6 PUFA) upregulates the expression of 15-PGDH by inhibiting miR-26a and miR-26b in human cholangiocarcinoma cells. We show that 15-PGDH is a bona fide target of miR-26a and miR-26b. Our findings provide novel evidence for ω-3 PUFA-regulated miR-26a/b and 15-PGDH cascade and support ω-3 PUFA as a nontoxic therapeutic agent for the treatment of human cholangiocarcinoma.

Materials and Methods

Materials

Docosahexaenoic acid (DHA) and AA were purchased from Cayman Chemical. mir-26a and mir-26b lentiviral particles were purchased from GeneCopoeia. 15-PGDH 3’ untranslated region (UTR)–luciferase reporter was obtained from ORIGENE. Rabbit polyclonal antibody against 15-PGDH was purchased from Cayman chemical. Rabbit polyclonal antibody against c-myc was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibodies against CTDSPL and CTDSP1 were purchased from Abcam. Mouse monoclonal antibodies against β-actin were purchased from Sigma-Aldrich. siRNA against 15-PGDH was synthesized by ORIGENE. NOD CB17-prkdc/scid mice were purchased from The Jackson Laboratory and maintained in Tulane transgenic mouse facility according to the protocol approved by the American Association for Accreditation of Laboratory Animal Care. All primers used in this study were synthesized by Integrated DNA Technologies (Supplementary Table S1). All chemical reagents were analytic grades (Sigma).

Cell culture

Two human cholangiocarcinoma cell lines, CCLP1 and TFK-1, were used in this study. TFK-1 cells (37) were obtained from the Japanese Cancer Research Resources Bank where the cell line was tested and authenticated by DNA fingerprinting, isozone detection, cell vitality assay, and mycoplasma detection. Authentication of CCLP1 cells (38) was based on morphologic features, immunohistochemistry, ultrastructural analysis, and cytogenetic analysis, and the cells were cultured as we previously described (14, 17, 19). The TFK-1 and CCLP1 were maintained in RPMI-1640 and DMEM culture medium, respectively. All culture medium were supplemented with 10% (v/v) FBS and 1% (v/v) penicillin and streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. All cell lines were used at low passage number not exceeding 30 passages.

Stable cell lines

CCLP1 and TFK-1 cells were transfected with Fat-1 expression plasmid or control vector pCDNA3 and then maintained in complete culture medium with 0.2 μg/ml puromycin (Life Technology). CCLP1 and TFK-1 cells were also infected with miR-26a/b lentivirus or miRNA-scramble control and the cells were maintained in culture medium with 0.2 μg/ml Geneticin (Life Technologies). Medium is replaced every 3 days for 2 to 4 weeks until outgrowth of resistant cells. The resistant cells were harvested and maintained in culture media with selection agents for further use.

Gene-expression analysis

Total RNA was extracted according to the TRIzol Reagent method (Life Technologies). mRNA levels were quantified by using the RT² SYBR Green qPCR Kit (QIAGEN); GAPDH is measured as a reference gene. mRNA levels were quantified by using the miScript Primer Assays Kit (QIAGEN). U6 is measured as a reference gene. Primers used are listed in Supplementary Table S1. For Western blotting analyses, whole-cell lysate were prepared by using RIPA lysis buffer with protease inhibitor cocktail tablets (Roche Diagnostics). Cellular proteins were separated by SDS-PAGE electrophoresis and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was blocked by PBS-T (0.5% Tween 20 in PBS) containing 5% nonfat milk for 1 hour at room temperature, and then incubated with individual primary antibodies in PBS-T containing 5% nonfat milk for 2 to 5 hours at room temperature with the dilutions specified by the manufacturers. Following three washes with PBS-T, the membranes were incubated with IRDye 680LT/IRDye 800CW secondary antibodies (LI-COR Biosciences) in PBS-T for 1 hour room temperature. The membranes were then washed with PBS-T and the protein bands were visualized by using the Odyssey infrared imaging system (LI-COR Biosciences).

Dual-luciferase reporter assay

Cells were cotransfected with luciferase reporter (15-PGDH promoter-luciferase, 15-PGDH 3’UTR-luciferase) and pRL-TK (Promega). pRL-TK provides the constitutive expression of Renilla luciferase that was used as an internal control. Seventy-two hours after transfection, cells were collected and passively lysed. Luciferase activities in the extracts were measured by DLRready Centro XS³ LB960 luminometer with the use of Dual-Luciferase Reporter Assay Kit (Promega). Luciferase activity was measured against Renilla luciferase activity for transfection efficiency.

Chromatin immunoprecipitation assay

Cells were cross-linked by 1% formaldehyde for 10 minutes. Chromosome DNA was extracted according to the protocol provided by SimpleChIP Assay Kits (Cell Signaling Technology) and precipitated with specific c-myc Rabbit polyclone antibody. Rabbit polyclone antibody Histone 3 was used as positive control whereas Rabbit IgG was used as negative control. Regular PCR procedure (5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C, ended by 10 minutes at 72°C) was adopted to amplify c-myc–binding site sequence. Primers were listed in Supplementary Table S1.
Cell proliferation assay
A total of 5 × 10^3 cells were plated in each well of 96-well plates and synchronized in G_0 phase by serum deprivation. Growth arrest was released by adding 2% serum. WST-1 reagent (Roche Diagnostics) was used to detect cell proliferation rate according to the manufacturer’s instructions. Each point in cell growth curve represents the mean of three independent normalized OD_{450} readings.

Colony-forming assay
A total of 1 × 10^3 cells were plated in 10-cm dish and allowed to grow for 14 days. The colonies were stained with crystal violet (Amresco). The colonies in each dish were counted.

TUNEL assay
Cells (1 × 10^4 per well) were seeded in 8-well chamber slide and cultured overnight. Then, the cells were fixed by 4% formaldehyde in PBS for 25 minutes. The cell apoptosis on the slide was detected according to the protocol of Deadend colorimetric TUNEL system (Promega).

Xenograft tumor study in SCID mice
SCID mice were injected s.c. at the axillary area with indicated groups of CCLP1 cells (1 × 10^7 cells in 100 μL of PBS). The mice were closely monitored for tumor growth and sacrificed 35 days after inoculation to recover the tumors. The tumor volume was measured and calculated by using the formula: larger diameter × (smaller diameter)^2/2. RNA was extracted from recovered tumor tissues using TRIzol Reagent (Life Technologies) to measure the level of miR-26a. Proteins from the tumor tissues were extracted by using NP-40 lysis buffer for Western blotting analysis.

Intrahepatic tumor growth via splenic injection
General anesthesia in mice was induced by Fluriso (Vetone). The abdominal cavity was opened by a 0.5-cm left sided transverse incision. The liver was exposed and an incision was made in the right lobe of the liver. A total of 5 × 10^6 cells were injected into the spleen. The host liver was sutured and the incision was closed. The mice were monitored daily and the tumor size was recorded. The tumors were excised after the mice were sacrificed and the tumor volume was calculated by using the formula: larger diameter × (smaller diameter)^2/2.
laparotomy. The spleen was identified, and 1 x 10^5 cells (with or without 15-PGDH knockdown) in a total volume of 100 μL PBS were injected into the spleen. After tumor cell inoculation, the spleen was resected and the abdominal cavity was closed by a running 3/0 braided silk suture (CP medical). The mice were i.p. injected with 200 μL DHA (0.5 mg/mL, dissolved in BSA solution) or BSA control every 2 days (starting 2 days after surgery). Two weeks after DHA treatment, the mice were sacrificed and the livers were removed to document tumor growth parameters [tumor volume was calculated by using the formula: larger diameter x (smaller diameter)^2/2].

Statistical analysis

Results are presented as mean ± SE from a minimum of three replicates. Differences between groups were evaluated by SigmaPlot statistical software with unpaired analysis of the Student t test and

Figure 2.

ω-3 PUFAs upregulate the expression of 15-PGDH in cholangiocarcinoma. A, putative miR-26a- and miR-26b-binding site in normal 3'UTR or mutated 3'UTR of 15-PGDH mRNA. B, DHA, but not AA, decreases the levels of miR-26a and miR-26b in cholangiocarcinoma cells. CCLP1 or TFK-1 cells were synchronized by serum deprivation and then maintained in serum-free medium containing 50 μM DHA or AA for 12 hours. *P < 0.05; **P < 0.01. C, Fat-1 expression decreased miR-26a and miR-26b levels in cholangiocarcinoma cells. **P < 0.05; ***P < 0.001. D, the levels of miR-26a or miR-26b in cholangiocarcinoma cells infected with respective lentiviral vectors. CCLP1 or TFK-1 cells were transfected with 15-PGDH 3'UTR luciferase reporter vector or mutated construct. *P < 0.05; **P < 0.01. G, miR-26a expression prevented ω-3 PUFA–induced 15-PGDH expression. CCLP1 and TFK-1 cells overexpressing miR-26a were treated with or without 50 μM DHA for 12 hours. Cellular proteins were analyzed by Western blotting using 15-PGDH antibody (β-actin was measured as a reference gene).
Results

ω-3 PUFA induces 15-PGDH expression in human cholangiocarcinoma cells

We compared the effect of ω-3 PUFA (DHA) versus ω-6 PUFA (AA) on 15-PGDH expression in human cholangiocarcinoma cell lines (CCLP1 and TFK-1). Although DHA treatment increased the level of 15-PGDH protein, AA treatment exhibited no effect (Fig. 1A and Supplementary Fig. S1). In separate experiments, we stably transfected CCLP1 and TFK-1 cells with vector expressing the Fat-1 gene (which encodes a C. elegans ω-3 fatty-acid desaturase converting ω-6 to ω-3 fatty acids; ref. 39). Overexpression of the Fat-1 gene was also found to increase 15-PGDH protein expression; the effect of Fat-1 gene transfection on 15-PGDH is comparable with DHA treatment of control cells (Fig. 1B). In addition, the levels of 15-PGDH mRNA were also elevated in DHA-treated or Fat-1 overexpressed cells (Fig. 1C).
DHA treatment or Fat-1 expression did not alter 15-PGDH promoter activity, as reflected by the 15-PGDH promoter luciferase assay (Fig. 1D).

ω-3 PUFAs suppress miR-26a/b and prevent their targeting of 15-PGDH in cholangiocarcinoma cells

As ω-3 PUFA increased 15-PGDH protein and mRNA levels without induction of 15-PGDH promoter activity, we reasoned that ω-3 PUFA might regulate 15-PGDH gene expression through a posttranscriptional mechanism. We directed our attention to microRNAs, which could potentially bind to 15-PGDH 3′UTR. Sequence analysis identified four conserved microRNAs (miR-26a, miR-26b, miR-1297, and miR-4465) that are complementary to the 15-PGDH 3′UTR (Fig. 2A and Supplementary Fig. S2). Among these four microRNAs, miR-26a and miR-26b were found to be highly expressed in cholangiocarcinoma cells relative to the other two (miR-1297 and miR-4465; Supplementary Fig. S2). We next performed qRT-PCR analysis to determine whether ω-3 PUFA might alter the expression of these microRNAs. As shown in Fig. 2B and C, DHA treatment or Fat-1 overexpression decreased the levels of miR-26a and miR-26b, but not the other two microRNAs (miR-1297 and miR-4465). These findings suggest that ω-3 PUFA may induce 15-PGDH expression through alteration of miR-26a and/or miR-26b.

To further determine the effect of miR-26a and miR-26b on 15-PGDH, we infected human cholangiocarcinoma cells with lentivirus particles carrying miR-26a (green) or miR-26b gene (red; Fig. 2D); these cells were then analyzed for 15-PGDH protein expression. As shown in Fig. 2E, overexpression of miR-26a or miR-26b significantly reduced 15-PGDH protein in both CCLP1 and TFK-1 cells and the effects were reversed by anti-miR-26. We next measured the 15-PGDH 3′UTR luciferase reporter activities in miR-26a or miR-26b overexpressed or control cells. As shown in Fig. 2F, miR-26a or miR-26b overexpression decreased the 15-PGDH 3′UTR luciferase reporter activity; this effect was abolished when the miR-26a/b–binding sites were mutated. These results establish 15-PGDH as a direct target of miR-26a/b. Accordingly, we observed that overexpression of miR-26a or miR-26b prevented DHA-induced 15-PGDH protein accumulation (Fig. 2G). Taken together, our findings suggest that ω-3 PUFAs induce 15-PGDH protein accumulation through suppression of miR-26a/b in human cholangiocarcinoma cells.

C-myc is implicated in ω-3 PUFA-induced suppression of miR-26a/b

miR-26a/b are located in the introns of CTDSPPs (carboxy-terminal domain RNA polymerase II polypeptide A small
phosphatase) gene family (illustrated in Fig. 3A; refs. 40, 41).

Given that the expression of miR-26a/b is reported to be concomitant with their host genes (40), we measured the mRNA level of CTDSPs (CTDSPL and CTDSP1) in cholangiocarcinoma cells treated with ω-3 PUFA. Our data showed that the ω-3 PUFA DHA suppressed the expression of both CTDSPL and CTDSP1, whereas the ω-6 PUFA AA had no effect (Fig. 3B). The pattern of CTDSPL and CTDSP1 alterations appears to be similar to their intronic microRNAs, suggesting that miR-26a/b and their host genes are coregulated by ω-6 PUFA in cholangiocarcinoma cells.

The expression of CTDSPs is well known to be associated with the transcription factor c-myc. Given that c-myc is a downstream gene of Wnt signaling (42–44) and that ω-3 PUFA suppresses the Wnt pathway (45, 46), we sought to further examine whether c-myc might be implicated in ω-3 PUFA-mediated suppression of CTDSPs/miR-26s. Our data showed that DHA treatment decreased c-myc along with reduction of CTDSPs/miR-26s. Our results showed that DHA treatment decreased c-myc along with reduction of CTDSPs/miR-26s. Chromatin immunoprecipitation (ChIP) assay showed that c-myc was associated with the promoters of the CTDSPL/miR-26a and CTDSP1/miR-26b gene clusters (Fig. 3E). These findings suggest that ω-3 PUFA regulates the expression of miR-26a/b at least in part through c-myc.

Figure 5.
ω-3 PUFAs induce 15-PGDH and inhibit cholangiocarcinoma growth in vivo. CCLP1 cells (with Fat-1 overexpression, miR-26a overexpression, Fat-1/miR-26a coexpression, or control vector cells) were inoculated into SCID mice (n = 6). Tumors growth was monitored and recovered 35 days later. Overexpression of Fat-1 inhibited tumor growth in vivo; this effect was reversed by overexpression of miR-26a. Overexpression of miR-26a alone was found to enhance tumor growth in vivo. A, gross photograph of tumors recovered from SCID mice. B, bar graphs showing the average volume of recovered tumors and the average miR-26a expression levels in the recovered tumors. The volume of tumor was calculated as described in Materials and Methods; the level of miR-26a was measured by real-time PCR. Results were normalized to the control group. Data, mean ± SE; *, P < 0.05; **, P < 0.01; C, representative Western blot analysis for 15-PGDH in recovered tumor tissues. β-Actin was measured as a reference gene.
Fat-1 of 15-PGDH in CCLP1 cells prevents of cholangiocarcinoma growth

Overexpression of miR-26a prevents Fat-1–induced inhibition of cholangiocarcinoma growth

To further determine the role of miR-26/15-PGDH in ω-3 PUFAs-induced inhibition of cholangiocarcinoma cell growth, we evaluated the growth parameters of tumor cells overexpressing Fat1 and/or miR-26a. As shown in Fig. 4A and B, overexpression of miR-26a abolished Fat-1–induced inhibition of CCLP1 cell growth and colony formation, in vitro. TUNEL assay showed that Fat-1–induced CCLP1 cell apoptosis was partially reversed by miR-26a overexpression (Supplementary Fig. S3; this result is consistent with our previous report that ω-3 PUFAs inhibit cholangiocarcinoma predominantly through induction of apoptosis; ref. 19). Our further Western blotting analysis confirmed that miR-26a overexpression attenuated Fat-1–induced induction of 15-PGDH (Fig. 4C).

We then inoculated CCLP1 cells with or without Fat-1 and/or miR-26a overexpression subcutaneously into SCID mice to monitor tumor growth in vivo. Although Fat-1 expression inhibited xenograft tumor growth, overexpression of miR-26a enhanced tumor growth and offset the inhibitory effect of Fat-1 (Fig. 5A and B). Western blotting analysis using the recovered xenograft tumor tissues confirmed that miR-26a overexpression attenuated Fat-1–induced induction of 15-PGDH in vivo (Fig. 5C).

Knockdown of 15-PGDH prevents ω-3 PUFAs–induced inhibition of cholangiocarcinoma growth

To further determine the role of 15-PGDH in ω-3 PUFA–induced inhibition of cholangiocarcinoma growth, we constructed cells with Fat-1 overexpressing plus 15-PGDH knockdown. By using siRNA approach, we were able to satisfactorily reduce 15-PGDH protein in normal or Fat-1–expressed CCLP1 cells (Fig. 6A). We observed that knockdown of 15-PGDH reversed Fat-1–induced inhibition of CCLP1 cell proliferation and colony formation, in vitro (Fig. 6B and C). We next performed in vivo experiments to evaluate the effect of ω-3 PUFAs and 15-PGDH on cholangiocarcinoma growth in SCID mice. We observed that administration of exogenous DHA to SCID mice significantly decreased tumor growth when the mice were inoculated with control vector tumor cells and that 15-PGDH knockdown reversed DHA effect in vivo (Fig. 7). These findings provide in vitro and in vivo evidence for an important role of 15-PGDH in ω-3 PUFA–induced inhibition of cholangiocarcinoma cell growth.

Discussion

This study provides the first evidence that ω-3 PUFAs upregulate the expression of 15-PGDH by inhibiting miR-26a and miR-26b and that these effects contribute to ω-3 PUFA–induced inhibition of cholangiocarcinoma growth. Our findings support that ω-3 PUFA may be used as a nontoxic adjuvant therapeutic agent for the treatment of human cholangiocarcinoma. The significance of the study is further underscored by the fact that cholangiocarcinoma is a highly malignant human cancer currently with no effective therapy.

Previous studies have shown that ω-3 PUFAs inhibit the growth of tumor cells, although they are significantly less toxic toward normal cells (47, 48). For cells of biliary origin, we have shown that ω-3 PUFAs inhibit the growth of cholangiocarcinoma cells but not primary biliary epithelial cells (19). Thus, ω-3 PUFA may represent a nontoxic therapeutic agent for treatment of human cholangiocarcinoma. Several mechanisms for ω-3 PUFA as a cancer therapeutic agent have been documented (49). Our previous study has shown that ω-3 PUFAs inhibit cholangiocarcinoma cell growth in part through inhibition of Wnt/β-catenin and COX-2 signaling pathways.
Another novel aspect of this study is the illustration of miR-26a/b as a key factor linking ω-3 PUFA to 15-PGDH. We show that ω-3 PUFA inhibits the expression of miR-26a/b, thus leading to 15-PGDH protein accumulation. Direct targeting of 15-PGDH by miR-26a/b was demonstrated by the observations that miR-26a/b inhibits 15-PGDH 3′UTR luciferase reporter activity and that miR-26a/b overexpression prevents ω-3 PUFA-induced 15-PGDH protein accumulation. We noted that knockdown of 15-PGDH did not reverse cholangiocarcinoma growth as potently as miR-26a/b overexpression; this aspect may be explained by the facts that miR-26a enhance Wnt/β-catenin signaling via inhibiting GSK-3β, and that GSK-3β is another target of ω-3 PUFA (19, 50). Thus, the data presented in this study, along with our previous findings, suggest that there are two targets, 15-PGDH and GSK-3β, which can be regulated by ω-3 PUFA/miR-26s in human cholangiocarcinoma cells. The interplays between PGE₂ and Wnt/β-catenin signaling pathways and their regulation by ω-3 PUFA are illustrated in Supplementary Fig. S4.

Although c-myc is a downstream oncogene of Wnt/β-catenin signaling, it is also the cofactor regulating the gene clusters formed by miR-26a/b and their host CTDSPL genes (40–43). Our data presented in this study suggest that ω-3 PUFA suppress miR-26a and miR-26b by inhibiting c-myc, through regulation of their host genes CTDSPLs. The latter assertion is further supported by the ChIP assay showing that c-myc is associated with the promoters of the CTDSPL/miR-26a and CTDSPL1/miR-26b gene clusters and by the observation that overexpression of c-myc prevents ω-3 PUFA-induced reduction of CTDSPLs/miR-26s.

In summary, this study provides novel evidence for induction of 15-PGDH by ω-3 PUFA via suppression of miR-26s in human cholangiocarcinoma cells. Our findings further support the use of ω-3 PUFA as nontoxic adjuvant therapeutic agent for the treatment of human cholangiocarcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Development of methodology: L. Yao, K. Song
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Grant Support
This works is supported by grants from NCI and NIDDK (R01 CA134568, R01 CA102325, R01 CA106280, and R01 DK077776, T. Wu).

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Received August 29, 2014; revised January 10, 2015; accepted January 26, 2015; published OnlineFirst February 17, 2015.
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