Oncomir MicroRNA-346 Is Upregulated in Colons of Patients With Primary Sclerosing Cholangitis

Agnieszka Kempinska-Podhorodecka, PhD1, Małgorzata Blatkiewicz, PhD1, Ewa Wunsch, MD, PhD2, Łukasz Krupa, MD3, Krzysztof Gutkowski, MD, PhD3, Piotr Milkiewicz, MD, DSc2,4 and Małgorzata Milkiewicz, PhD1

INTRODUCTION: Primary sclerosing cholangitis (PSC) is a cholestatic liver disorder that is frequently associated with ulcerative colitis (UC). Patients with PSC and UC (PSC-UC) have a higher risk of colorectal neoplasia compared with patients with UC. The oncogenic properties of microRNA-346 (miR-346) have been recently reported. We investigated the expression of miR-346 and its 2 target genes, the receptor of vitamin D (VDR), and the tumor necrosis factor-α (TNF-α), which are known to modulate carcinogenesis.

METHODS: Ascending and sigmoid colon biopsies were obtained from patients with PSC, PSC and UC (PSC-UC), UC, and healthy controls (n=10 in each group). Expressions of VDR, TNF-α, 18S RNA, p27Kip1, miR-346, and reference microRNA, miR-191, were evaluated by real-time PCR using human TaqMan Gene Expression and TaqMan MicroRNA Assays. Functional studies with miR-346 mimic and inhibitor were conducted in HepG2 and Caco-2 cells. The effect of ursodeoxycholic acid on miR-346 expression was examined in Caco-2 cells.

RESULTS: An increased expression of miR-346 in the ascending colon of PSC-UC was observed (P<0.001 vs all groups). In patients with UC, an exceptionally low colonic expression of miRNA-346 was accompanied by the extensive upregulation of VDR and TNF-α genes. A functional in vitro analysis demonstrated that inhibition of miR-346 resulted in the upregulation of VDR and TNF-α, whereas the induction of miR-346 activity suppressed VDR, TNF-α, and p27Kip1.

DISCUSSION: The upregulation of miRNA-346 in the colon of patients with PSC may be responsible for the disturbance of VDR and TNF-α signaling pathway, which could result in an inadequate suppression of neoplasia.
target genes (11). Recently, miR-346 has been reported as an oncogenic miRNA (oncomiR) in numerous cancers, including prostate, lung, breast, and liver (12–14), but no data exist on its potential role in colorectal neoplasia.

MicroRNA-346 inhibits, among other target genes, the expression of the vitamin D receptor (VDR) via direct binding to a conserved target site within the 3’UTR of the VDR transcript (15). VDR is a nuclear receptor that mediates the biological activities of 1,25-dihydroxyvitamin D and is abundantly expressed in the epithelial cells of the gastrointestinal tract. Apart from the control of calcium homeostasis, it modulates the autocrine–paracrine regulation of cell proliferation and differentiation. The antiproliferative effects of VDR have been demonstrated in a wide variety of cancer cell lines. Several lines of evidence suggest that VDR activation, which induces the expression of cycle inhibitor p27(kip1), may be protective against cancer (16,17), and low levels of vitamin D have been associated both with cancer and altered immune responses (18–20). A reduction in epithelial VDR was suggested to affect the gut mucosal barrier and contributes to the development of IBD (21). Moreover, the role of vitamin D in immune-mediated diseases seems to be closely associated with bacterial metabolism and chronic dysbiosis may trigger VDR dysfunction (16). Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine and a key player in the pathogenesis of many inflammatory and autoimmune diseases. Recently, it was reported that miR-346 can indirectly modulate TNF-α expression either by the inhibition of Bruton tyrosine kinase (Btk) expression, which is required for TNF-α production, or by inducing tristetraprolin, which destabilizes TNF-α transcript (22).

Given that patients with PSC have an increased risk of colorectal neoplasia in comparison to healthy subjects and patients with UC, we investigated the expression of miR-346 and its 2 target genes, including VDR and TNF-α, in human colonic biopsies of patients with PSC or with UC. Moreover, the effect of miR-346 on VDR, TNF-α, and cycle inhibitor p27kip1 expression was studied in vitro.

METHODS
Patient characteristics
This study included 20 patients with PSC who underwent routine surveillance colonoscopies. Based on histopathological evaluation, they were divided into groups: the PSC group, who had never been diagnosed with concomitant IBD (n = 10), and the PSC-UC group showing the macroscopic features of UC on colonoscopy, which were confirmed with histology examination (n = 10). In addition, 10 patients with UC and 10 healthy controls who underwent colonoscopies for various indications and showed neither macroscopic nor microscopic abnormalities in their colons were included in the study (Table 1). All patients with PSC were treated with ursodeoxycholic acid (UDCA) (15 mg/kg body weight), and patients with PSC-UC additionally received 5ASA (2–3 g/daily). Three biopsy specimens per patients were obtained from both the ascending colon and the sigmoid colon. Each patient gave informed consent before participating in this study. The research protocol was approved by the Ethics Committee of Pomeranian Medical University and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

RNA and miRNA expression analysis
Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendations. Expressions of specific genes were measured using human TaqMan Gene Expression Assays (TNF-α Hs00174128_m1 and p27kip1 Hs0015327_m1) or TaqMan MicroRNA Assays (miR-346: 478046_mir). 18SRNA (Hs99999901_s1) and miR-191 (477952_mir) were used as control for data normalization. The fluorescence data were analyzed by the 7500 Fast Real-Time PCR System with 7500 Software v2.0.2 (Applied Biosystems, Foster City, CA), and relative amounts of transcripts were calculated using the 2−ΔΔCt formula.

Western blot analysis
After electrophoresis, the proteins were blotted into polyvinylidene difluoride membranes under semi-dry transfer conditions (Thermo Scientific, Rockford, IL). After blocking with 5% milk, the following antibodies were used: anti-VDR (1:200, sc-13133; Santa Cruz Biotechnology, Dallas, TX), anti-TNF-α (1:500, ab183896; Abcam, Cambridge, United Kingdom), and secondary antibodies (Code: 115-035-146; Code: 111-035-003, Jackson ImmunoResearch, West Grove, PA). Protein loading was normalized to anti-GAPDH (1:5,000, sc-365062; Santa Cruz). Bands were visualized by chemiluminescence detection (Chemiluminescent HRP Substrate, Millipore, MA) and quantified using the MicroChem 2.0 System. Specific bands were quantified by densitometric analyses with GelQuant software (Jerusalem, Israel).

Immunohistochemistry
The paraffin sections of colon tissue after deparaffinization and antigen unmasking with citrate buffer were exposed to Normal Horse Serum to block unspecific binding (Vector Laboratories, Burlingame, CA). Anti-VDR (sc-13133; Santa Cruz) or anti-TNF-α (sc-52746; Santa Cruz) and biotinylated anti-mouse/anti-rabbit IgG (BA-1400, Vector Laboratories) were used as primary and secondary antibodies, respectively. Reactions were visualized using ABC Vectastain and DAB kits (Dako, Glostrup, Denmark). Tissue structures were visualized by Mayer hematoxylin staining (DAKO). The negative controls, in which the primary antibodies were omitted, were included in the study and uniformly demonstrated no reaction. Images were acquired with the ZEISS Axio Imager Z2 microscope.

Cell culture and transfection
HepG2 cells, as miR-346 was demonstrated to be highly expressed in that cell line (14), and human epithelial colorectal adenocarcinoma cells, Caco-2 (American Type Culture Collection), were transfected with an miR-346 inhibitor (Ambion Anti-miR miRNA Inhibitor, hsa-miR-346; ID: AM10238; Thermo Fisher Scientific, Waltham, MA) or mimic (mirVana miRNA mimic, hsa-miR-346; ID: MC10238; Thermo Fisher Scientific) according to the producer’s protocol using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, the cells were lysated, and RNA was isolated. Caco-2 cell lines were grown on 96-well plates and treated with different concentrations (50 or 150 μM) of UDCA (Sigma-Aldrich, UDCA ≥99%, ID: U5127-1G). Twenty-four hours after UDCA treatment, the cells were harvested for further molecular analysis.

Statistics
Results were statistically analyzed using the StatView Program (SAS Institute, Cary, NC). Comparisons between the groups were made using the nonparametric Mann–Whitney test. Correlations were assessed by nonparametric Spearman rank correlation...
controls; the miRNA-346 expression was very low (did not reach statistical significance). The expression of miRNA-346 was enhanced in patients with PSC, but it was 0.02 and 22-fold vs PSC-UC, 0.004 and 8-fold vs PSC-UC, 0.007; Figure 2b) and TNF-α protein level (19-fold vs controls, 0.0004; 20-fold vs PSC P = 0.02 and 22-fold vs PSC-UC, P = 0.02; Figure 2d). TNF-α mRNA and protein levels were similar to the control group values in the PSC and PSC-UC groups (Figures 2b, d).

In terms of VDR protein levels, both in the ascending and sigmoid colons of patients with PSC and PSC-UC, there was a high variability between patients, and the overall mean values were similar to controls (Figures 1c and 2c). On the other hand, in patients with UC, the VDR protein expression was significantly increased in the ascending and sigmoid colons, not only in comparison to controls (4.7-fold, P = 0.02 and 9.5-fold, P = 0.002, respectively) but also when compared with PSC (2.5-fold, P = 0.004 and 9-fold, P = 0.001, respectively) and PSC-UC (2.5-fold, P = 0.006 and 6-fold, P = 0.002, respectively; Figures 1c and 2c).

A significant negative correlation was observed between VDR protein and miRNA-346 in patients with UC in both parts of the colon (sigmoid colon: Rho = −0.5; P = 0.04; and ascending colon: Rho = −0.8; P = 0.02). Furthermore, VDR protein levels were correlated positively with TNF-α mRNA in the sigmoid colon of all the patients (PSC: Rho = 0.8; P = 0.07; PSC + UC: Rho = 0.9; P = 0.05 and UC: Rho = 0.7; P = 0.01) and the ascending colon of patients with UC (Rho = 0.9; P = 0.02).

The localization of VDR and TNF-α protein in control, PSC-UC, and UC in human intestinal tissue is presented in Figure 3.

Finally, to find whether miRNA-346 is directly involved in the regulation of TNF-α and VDR gene expression, we used antisense oligonucleotide molecules to block miR-346 activity. Transfection of the inhibitor targeting miR-346 into HepG2 cells effectively suppressed miR-346 (Figure 4a) and simultaneously induced TNF-α and VDR mRNA expression (6.6 ± 1.9 vs 1.0 ± 1.7 in controls, P = 0.01; and 32.7 ± 13.8 vs 1.4 ± 1.2 in controls, P = 0.01, respectively, Figure 4a). Similarly, transfection of the miR-346 inhibitor into Caco-2 cells effectively inhibited miR-346 expression (Figure 4b) and simultaneously enhanced the levels of TNF-α and VDR mRNA (3.3 ± 0.1 vs 1.2 ± 0.2 in controls, P = 0.002; and 4.4 ± 0.9 vs 1.1 ± 0.2 in controls, P = 0.003, respectively, Figure 4b).

In contrast, transfection of a modified double-stranded RNA that mimics endogenous miR-346 into Caco-2 cells resulted in upregulation of that miRNA expression (4.5-fold increase, P = 0.002 vs controls; Figure 4c) and led to the downregulation of TNFα and VDR mRNA (0.7 ± 0.1 vs 1.1 ± 0.2 in controls, P = 0.01; and 0.5 ± 0.2 vs 0.9 ± 0.1 in controls, P = 0.03, respectively, Figure 4c). These data demonstrate that miR-346 is implicated in the negative regulation of TNFα and VDR synthesis. Given that miR-346 may promote cell cycle by inhibition of p27^Kip1 via suppression of sFRP4 (23),

### Table 1. Demographic and laboratory features of analyzed subjects

| Parameters | Control (n = 10) | PSC (n = 10) | PSC-UC (n = 10) | UC (n = 10) |
|------------|----------------|-------------|----------------|------------|
| Sex (M/F)  | 6/4           | 8/2         | 7/3            | 2/8        |
| Age (mean ± SD) | 50 ± 4     | 30 ± 9      | 36 ± 17        | 43.4 ± 15  |
| Hb (mmol/L, mean ± SD) | —       | 13.5 ± 9.0  | 13.1 ± 1.8     | —         |
| Bilirubin (μmol/L, mean ± SD) | —       | 10.1 ± 3.6  | 25.3 ± 31.5    | —         |
| ALP (U/L, mean ± SD) | —       | 159 ± 108   | 303 ± 284      | —         |
| GGTP (U/L, mean ± SD) | —       | 302 ± 332   | 381 ± 187      | —         |
| ALT (U/L, mean ± SD) | —       | 103 ± 86    | 56 ± 67        | —         |

ALP, alkaline phosphatase; ALT, alanine aminotransferase; GGTP, gamma-glutamyl transferase; Hb, hemoglobin; PSC, primary sclerosing cholangitis; UC, ulcerative colitis.
we additionally analyzed the level of p27Kip1 in Caco-2 cells transfected with miR-346 mimics. Increased miR-346 expression contributed to the downregulation of p27Kip1 (0.4 ± 0.1 vs 1.1 ± 0.2 in controls, P = 0.0006; Figure 4c).

Figure 1. Expression of miRNA-346, TNF-α, VDR, and p27Kip1 in the ascending colon. In patients with PSC-UC, the expression of miRNA-346 (a) was increased, whereas the levels of VDR protein (c) and TNF-α protein (d) were not changed in comparison to controls. The expression cell cycle inhibitor p27Kip1 (e) was substantially inhibited both in patients with PSC-UC and PSC. In contrast, in patients with UC, the miRNA-346 expression (a) was drastically suppressed, and it was accompanied by the enhanced expression of TNF-α mRNA (b), VDR protein (c), and TNF-α protein (d), whereas the expression cell cycle inhibitor p27Kip1 (e) was comparable to control values. MicroRNA-191 served as reference microRNA. Results are representative of n = 10 independent experiments per group. Data are presented as median plus IQR. IQR, interquartile range; VDR, receptor of vitamin D.

DISCUSSION

The 2 main findings of this study were (i) a divergent expression of miR-346 in the colonic tissues of patients with PSC in comparison to patients with UC alone and (ii) miR-346-dependent modulation of TNF-α and VDR expression in Caco-2 cell line.

Our study demonstrates the substantial increase in miR-346 expression in the intestinal mucosa of patients with PSC, but the localizations of these changes were different depending on the
part of the examined colon. Hence, in patients with PSC-UC, miR-346 was upregulated in the ascending colon, whereas in patients with PSC without concurrent UC, the changes in miR-346 expression were observed in the sigmoid colon. It is known that the right proximal ascending colon is the predilection site for the development of colonic malignancies in patients with PSC-UC, and these patients tend to have more progressive tumors than patients with IBD without concomitant PSC (4). Of note, miRNA-346 was hardly detected in both the ascending and sigmoid colons of patients with UC. These results are in agreement with previous studies in which miRNA-346 expression in colonic biopsies was reported to be consistently downregulated in both quiescent and active UC in comparison to healthy controls (24,25).

The very low expression of miR-346 in both examined parts of the colonic tissue of patients with UC was associated with a very significant increase in TNF-α mRNA and protein levels. These data are consistent with previous reports, which showed independently either diminished expressions of miR-346 or increased levels of TNF-α in the colons of patients with UC (24–26). Based on the animal and human data, it was suggested that TNF-α promotes miR-346 expression (15). However, in our study, this was not the case as we did not observe the induction of miR-346 in the presence of a very high level of TNF-α transcript. In contrast, in the colonic tissues of patients with PSC, we demonstrated a negative association between miR-346 expression and TNF-α mRNA level. This observation can be supported by a report showing that miR-346 plays a crucial role in the control of the inflammatory responses as it acts as a negative regulator of TNF-α release (22). In activated macrophages and fibroblast-like

Figure 2. Expression of miRNA-346, TNF-α, and VDR in the sigmoid colon. In both patients with PSC and PSC-UC, the levels of miRNA-346 (a), TNF-α mRNA (b), VDR protein (c), and TNF-α protein (d) were comparable to the control values. In patients with UC, the miRNA-346 expression (a) was drastically suppressed, and it was accompanied by the enhanced expression of miRNA TNF-α (b), VDR protein (c), and TNF-α protein (d). MicroRNA-191 served as reference microRNA. Results are representative of n = 10 independent experiments per group. Data are presented as median plus IQR. IQR, interquartile range; PSC, primary sclerosing cholangitis; TNF-α, tumor necrosis factor-α; VDR, receptor of vitamin D.
synoviocytes isolated from the joint spaces of patients with rheumatoid arthritis, TNF-α secretion was substantially inhibited by miR-346 (22). Our observations from the functional studies in which the experimental inhibition of miR-346 in Caco-2 and HepG2 cells led to the enhanced expression of TNF-α are in line with these reports. Our study reveals a distinct profile of TNF-α expression in patients with PSC vs UC. In the sigmoid colon of patients with PSC

**Figure 3.** Immunohistochemical localization of VDR and TNF-α proteins in human intestinal tissue. Representative immunostaining of colonic biopsies from controls, patients with PSC, patients with PSC-UC, and patients with UC with anti-TNF-α antibodies (a–d and i–l) and anti-VDR antibodies (e–h and m–p). Brown staining indicates either VDR protein (red arrows), which is located typically in the epithelial cells, or TNF-α protein (black arrows), which is depicted in expanded apical portions of goblet cells. Nuclei were visualized by hematoxylin. Original magnification was 200×. PSC, primary sclerosing cholangitis; TNF-α, tumor necrosis factor-α; VDR, receptor of vitamin D.

**Figure 4.** Effect of miR-346 inhibition or activation on TNF-α and VDR expression in cell lines. Caco-2 cells were untransfected (control) or transfected with miR-346 inhibitor (a, b) or miRNA-346 mimic (c). Twenty-four hours after transfection, levels of miR-346, TNF-α, and VDR transcript were quantified by qRT-PCR. Suppression of miR-346 by miR-346 antisense molecules was confirmed in both cell lines. Inhibition of miR-346 led to the strong upregulation of TNF-α and VDR in both HepG2 (a) and Caco-2 (b) cell lines. Induction of miR-346 led to a significant downregulation of TNF-α, VDR, and p27kip1 in Caco-2 cells (c). Each experiment was repeated 4 times with similar results. Data are presented as mean ± SE. TNF-α, tumor necrosis factor-α; VDR, receptor of vitamin D.
both with or without concomitant UC, the levels of TNF-α mRNA and protein were comparable to control values in contrast to patients with UC where the expression of TNF-α was drastically increased. In the ascending colon, the level of TNF-α mRNA was enhanced in all patients with PSC. However, this occurred to a significantly lesser extent (more than 8-fold less) than in the colonic mucosa of patients with UC. Moreover, the analyses of the TNF-α protein level showed that in contrast to patients with UC, the colonic expression of this cytokine in all patients with PSC was comparable to controls. The observed differences between mRNA and protein levels of TNF-α in patients with PSC and PSC-UC may be a result of epigenetic regulation of TNF-α gene expression by miR-346. TNF-α is a pleiotropic cytokine with dual roles in cancer biology as it has either pro- or anti-cancer activities (27). These paradoxical roles depend on its local tissue concentration (28). Hence, TNF-α is cytotoxic to tumor cells at high levels, whereas it fuels tumor-promoting inflammation and angiogenesis at low levels. The responses mediated by TNF-α are transduced via type 1 (TNFR-1) and type 2 receptors. TNFR-1 has a death membrane domain and mediates apoptosis, but apart from this, it triggers the transcription of NF-kB and c-Jun, which are involved in cell proliferation and growth (29). The association between chronic inflammation and the risk of colorectal cancer remains controversial. Several recent meta-analyses showed no evidence of the association between the circulation level of TNF-α and the risk of colorectal cancer (30,31). Moreover, anti-TNF-α therapies for the treatment of IBD, which aim to reduce the level of this cytokine, have been suggested to have some undesirable effects, such as the induction of malignancy (32).

VDR signaling plays a critical role in maintaining the integrity of the mucosal epithelial barrier by suppressing the apoptosis of inflammation-induced cells (33,34). The data on mucosal VDR expression in patients with cholestasis are very limited. The VDR is known to be involved in the phenotypic features of PSC either by having an indirect impact on intestinal permeability or directly modulating of innate immunity. We previously demonstrated that VDR protein expression was decreased considerably in peripheral blood mononuclear cells and in the livers of patients with PSC, but its colonic expression has not been investigated (35). In this study, we found that the upregulation of miR-346 in the ascending colon of patients with PSC-UC and in the sigmoid colon of patients with UC where the expression of VDR and TNF-α was drastically increased. In Caco-2 cells transfected with miR-346 mimics, we observed a considerable suppression of both VDR and TNF-α mRNA. Our findings are in agreement with the previous reports demonstrating downregulation of VDR expression by miR-346 (15,36). Moreover, we noticed that upregulation of miR-346 activity in those cells was associated with decreased expression of p27kip, a tumor suppressor gene, which is known to be downregulated in several human cancers, including CRC (37–39). Thus, the increased miR-346 expression may be associated with the downregulation of p27kip in the ascending colon of patients with PSC and may contribute to the development and progression of colon cancer. To the best of our knowledge, the expression of both miR-346 and cell cycle inhibitor p27kip in the colon of patients with PSC has not been investigated before.

We observed that a barely detectable expression of miRNA-346 in both parts of the colon of patients with UC was associated with a substantial induction of VDR. Our in vitro functional studies in 2 cell lines (HepG2 and Caco-2) with single-stranded RNA molecule, which inhibits endogenous miRNA-346, confirmed the inhibitory effect of this microRNA on VDR and TNF-α transcript.

In summary, the present study provides novel insights into miRNA-346-dependent modulation of TNF-α and VDR expression. The evidence from this study implies that the substantial upregulation of miRNA-346 in the ascending colon of patients with PSC-UC may be accountable for the suppression of cellular protective responses modulated by VDR and TNF-α, which may lead to an inadequate suppression of neoplasia. In addition, the enormous reduction of miRNA-346 in both parts of the large intestine of patients with UC, which was accompanied by the extensive increase in VDR and TNF-α, supports this notion. Further investigation of the potential importance of colonic miRNA-346, including upstream factors, in pathophysiological mechanisms of CRC development in patients with PSC is necessary.

CONFLICTS OF INTEREST
Guarantor of the article: Agnieszka Kempinska-Podhorodecka, PhD.

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**Study Highlights**

**WHAT IS KNOWN**
- PSC increases the risk of colitis-associated CRC.
- miR-346 was reported as an oncogenic miRNA in numerous cancers.
- miR-346 modulates VDR and TNF-α gene, which are known to be protective against carcinogenesis.

**WHAT IS NEW HERE**
- Upregulation of colonic miRNA-346 in patients with PSC-UC in contrast to patients with UC alone.
- Complete suppression of miRNA-346 in UC was associated with the upregulation of VDR and TNF-α.
- The inhibitory effect of miRNA-346 on VDR and TNF-α transcript was confirmed in vitro.

**TRANSITIONAL IMPACT**
- Inadequate colonic expression of VDR and TNF-α in PSC may lead to colorectal neoplasia.
- Inhibition of miRNA-346 is a potential target for pharmacological intervention in PSC.

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