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Upregulation of mir-506 Leads to Decreased AE2 Expression in Biliary Epithelium of Patients with Primary Biliary Cirrhosis

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Abstract

Cl⁻/HCO₃⁻ anion exchanger 2 (AE2) participates in intracellular pH homeostasis and secretin-stimulated biliary bicarbonate secretion. *AE2/SLC4A2* gene expression is reduced in liver and blood mononuclear cells from patients with primary biliary cirrhosis (PBC). Our previous findings of hepatic and immunological features mimicking PBC in *Ae2*-deficient mice strongly suggest that decreased AE2 expression might be involved in the pathogenesis of PBC. Here we tested the potential role of hsa-microRNA 506 (miR-506) – predicted as candidate to target AE2 mRNA – for the decreased expression of AE2 in PBC. Real-time qPCR showed that miR-506 expression is increased in PBC livers *versus* normal liver specimens. *In situ* hybridization in liver sections confirmed that miR-506 is upregulated in the intrahepatic bile ducts of PBC livers compared with normal and primary-sclerosing-cholangitis livers. Precursor-mediated overexpression of miR-506 in SV40-immortalized normal human cholangiocytes (H69 cells) led to decreased AE2 protein expression and activity, as indicated by immunoblotting and microfluorimetry, respectively. Moreover, miR-506 overexpression in 3D-cultured H69 cholangiocytes blocked the secretin-stimulated expansion of cystic structures developed under the three-dimensional conditions. Luciferase assays and site-directed mutagenesis demonstrated that miR-506 specifically may bind the 3'UTR region of AE2 mRNA and prevent protein translation. Finally, cultured PBC cholangiocytes showed decreased AE2 activity together with miR-506 overexpression compared to normal human cholangiocytes, and, transfection of PBC cholangiocytes with anti-miR-506 was able to improve their AE2 activity.

Conclusion—miR-506 is upregulated in cholangiocytes from PBC patients, binds the 3'UTR region of AE2 mRNA and prevents protein translation, leading to diminished AE2 activity and impaired biliary secretory functions. In view of the putative pathogenic role of decreased AE2 in PBC, miR-506 may constitute a potential therapeutic target for this disease.

Keywords

AE2; miRNAs; PBC; cholangiocytes

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No conflicts of interest exist.

Introduction

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease of unknown etiopathogenesis that mainly affects middle-age women.^{1–4} PBC livers exhibit nonsuppurative cholangitis with portal infiltrates and destruction of intralobular bile ducts effected by autoreactive T cells. A disease hallmark is the development of serum anti-mitochondrial autoantibodies (AMA), which together with other autoimmune phenomena are encountered in most PBC patients. Nevertheless, immunosuppressive agents show little therapeutic efficacy, while daily administration of ursodesoxycholic acid (UDCA), the only FDA-approved treatment for PBC, improves the prognosis in a majority of patients when started in early stages of the disease.^{1,5–7}

Among its multiple effects, which include poorly defined immunomodulatory properties, the hydrophilic bile acid UDCA is known to induce bicarbonate-rich hypercholeresis in humans.^{1,6,7} Interestingly, PBC patients who had not yet initiated the treatment with UDCA were shown to exhibit impaired biliary bicarbonate secretion in response to secretin administration, and this defect was restored in patients under UDCA therapy.⁸ As smartly illustrated by the bicarbonate-umbrella hypothesis, secretin-stimulated biliary bicarbonate secretion may be crucial in humans to prevent that the biliary epithelium becomes injured by hydrophobic bile acids.^{9,10} Secretin-stimulated biliary bicarbonate secretion is mediated by the $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger 2 (AE2),^{11–13} a widely expressed protein involved in hydroionic fluxes and intracellular pH homeostasis, which in the biliary epithelium is located on the apical surface of lining cholangiocytes.¹⁴ In cholangiocytes of PBC patients, both the expression of AE2 and the level of exchange activity following stimulation with cAMP (the second messenger of secretin signaling) are decreased.^{15,16} Of interest, the observed restoration of the secretin response in PBC patients under treatment with UDCA appeared to run parallel with increased expression of AE2 in PBC livers.^{8,15} These previous data supported the hypothesis that AE2 dysfunctions may have an important pathogenic role in PBC.¹⁷ In fact, common genetic variations of the *AE2/SLC4A2* gene have been associated with disease susceptibility and/or progression and the AMA status among PBC patients.^{18–20} Additional evidence for a pathogenic role of AE2 dysregulation was recently obtained with our *Ae2_{a,b}*-deficient mice, a model that develops biochemical, histological, and immunologic alterations that recapitulate many PBC features (including development of serum AMA).²¹ Thus, while the deficient expression of AE2 in cholangiocytes of patients with PBC appears to be involved in the pathogenesis of the disease, the mechanisms responsible for AE2 downregulation remain unclear.

MicroRNAs (miRNA) are a subclass of small, non-coding RNAs that have recently attracted a lot of attention because of their ability to post-transcriptionally regulate the expression of numerous genes into their encoded proteins.^{22–24} Moreover, abnormal protein expression contributing to the pathogenesis of a variety of diseases has increasingly been recognized to be due to alterations of specific miRNAs involved in regulating those proteins. Indeed, we reported that miR15-A is downregulated in cholangiocytes of patients with polycystic liver diseases leading to increased expression of CDC25A, a key regulator of the cell cycle, and accounting for the benign cholangiocyte hyperproliferation that results in hepatic cystogenesis.²⁵ And in patients with PBC, global changes in miRNA expression were recently found in the liver when microarray analyses were carried out.²⁶ With this background, here we tested the hypothesis that downregulation of AE2 in cholangiocytes of PBC patients might result from altered miRNA expression.

Our results support the conclusion that miR-506, the levels of which were reported to be increased in the liver of PBC patients,²⁶ is particularly overexpressed in the cholangiocytes

of these patients, binds directly to the 3'UTR region of AE2 mRNA inhibiting the protein translation, and resulting in decreased AE2 activity. Moreover, inhibition of miR-506 in cultured PBC cholangiocytes increases their AE2 activity. In view of the putative pathogenic role of decreased AE2 in PBC, miR-506 may therefore constitute a potential therapeutic target for this disease.

Materials and Methods

In Silico Predictions and In Situ Hybridization

According to the MicroCosm Targets resource (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>) that uses the miRBase database,²⁷ miR-506 was predicted to potentially target the 3'UTR region of human AE2 mRNA,²⁸ with base complementarities to the sequence CCCUGCAGUAAAGUGCUUUG within that 3'UTR region (see inset in Fig. 1A). Interestingly, miR-506 was one of the miRNAs encountered to be overexpressed in PBC livers when using a microarray.²⁶ We therefore carried out locked nucleic acid-based – *in situ* hybridization (LNA-ISH) analysis for miR-506 in sections of PBC and control liver tissue, as described in the online Supporting Information.

Primary Cholangiocytes and Biliary Cell Line

We used the H69 cholangiocyte cell line (a gift from Dr. D. Jefferson, Tufts University, Boston, MA), a well characterized SV40-transformed human bile duct epithelial cell line originally derived from a normal liver harvested for transplantation.²⁹ Also we used primary cultures of both PBC and normal human cholangiocytes isolated according to an original straightforward procedure described in the online Supporting Information. By using this procedure pure differentiated cholangiocytes (positive for cytokeratin-7 and 19, cystic fibrosis transmembrane conductance regulator, AE2 and aquaporin 1) were obtained (cf. Supporting Information and Supplementary Fig. 1).

Western Blot

H69 human cholangiocytes were transfected with 50 nM (final dilution) of either miR-506 precursor oligonucleotides, pre-miRNA negative control (both from Applied-Biosystems), or vehicle, using the siPORT NeoFX Transfection Agent (AM-4511, Applied-Biosystems). After 48 hours, changes in the protein expression of AE2 or CK19 were detected as described in the Supporting Information.

Luciferase Reporter Constructs and Assay

A 175-bp DNA amplicon of the 3'UTR region of human AE2 mRNA with the miR-506 target site was obtained by RT-PCR using specific oligonucleotides (forward 5'-CCCAAGCTTCCGCCACCGAGGG ACAGC-3' and reverse 5'-GACTAGTAGGTGGGGGCCAAAGCAC-3'). Subcloning of this fragment into the pMIR-REPORT Luciferase vector (Applied-Biosystems) resulted in the CMV-driven expression construct Luc-AE2-3'UTR. The mutated reporter construct Luc-mut-AE2-3'UTR was then obtained through site-directed mutagenesis of the putative miR-506 target site (wildtype 5'-CAGTAAAGTGCTTTG-3' → mutated 5'-TGATGAAGGGCTG CG-3'). H69 human cholangiocytes were cotransfected with either the wildtype or the mutated reporter construct together with miR-506 precursor oligonucleotides using the FuGENE-HD Transfection Reagent (Promega). Briefly, 3 µL of FuGENE were added to 97 µL of Opti-MEM and incubated for 5 minutes at room temperature. 50 nM (final dilution) of miR-506 precursor oligonucleotides (or pre-miRNA negative control) were added to the FuGENE/Opti-MEM mixture, incubated again for 15 minutes, and applied to the human cholangiocytes under

suspension. The luciferase activity was assessed 24 hours after transfection using the Luciferase Assay Kit E151A (Promega) in a NOVOstar Apparatus (BMG Labtech). Luciferase activity was normalized to TK Renilla construct as previously reported.³⁰

Assessment of AE2 Anion Exchange Activity

H69, PBC and normal human cholangiocytes were examined for their $\text{Cl}^-/\text{HCO}_3^-$ anion exchange activity (AE2 activity)¹² by microfluorimetry¹³ (cf. Supporting Information). Experiments were carried out in cells 48 hours after their transfection with 50 nM (final dilution) of either pre-miR-506, pre-miR negative control, commercial anti-miR-506 oligonucleotides (all from Applied-Biosystems) or vehicle.

Determination of miR-506 Expression Levels

Total RNA was isolated from both freshly cultured cholangiocytes and whole liver tissue with TRI-Reagent (Sigma). Aliquots (200 ng) were reverse-transcribed into cDNA using the TaqMan MicroRNA Reverse Transcription Kit and commercial miR-specific primers (Applied-Biosystems) in a total volume of 15 μL . The expression levels of four particular miRNAs (i.e. miR-506, miR-149-3p, miR-765 and miR-944) were determined by real-time quantitative PCR (qPCR) according to the TaqMan MicroRNA Assay protocol in a StepOne Plus Apparatus. For each miRNA, we employed 1.33 μL of the respective cDNA reaction as template and carried out qPCRs under the following conditions: 95°C for 10 minutes, and 45 cycles of both 95°C for 15 seconds and 60°C for 60 seconds. Data were analyzed by using the comparative Ct method and normalized with the expression of the Z-30 small nuclear RNA control (Applied-Biosystems).³¹ The control group was related to 100% of expression. Liver tissue samples were obtained from the University Clinic of Navarra and the experiments were approved by the University of Navarra Institution Review Board.

3D Culture of H69 Cholangiocytes

3D-cultured H69 cholangiocytes form cystic structures which expand over time as a consequence of fluid secretion.³² Briefly, confluent H69 cholangiocytes were scrapped in enriched DMEM-Ham's F-12 medium, transferred to a 50 mL Falcon tube at 37°C and let to stand during 2 hours for spontaneous recircularization. After a series of sequential filtrations through 100 and 40 μm meshes, H69 cystic structures ranging from 40 to 100 μm were seeded and grown between two layers of type I rat collagen (1.5 mg/mL; BD Biosciences) in enriched DMEM-Ham's F-12 medium 24 hours at 37°C in the presence of either pre-miR-506 or pre-miR-control (50 nM each)³³ or just vehicle. H69 cystic structures were then monitored for their expansion in response to 1 μM secretin (Bachem, Torrance, CA) for 30 minutes in enriched DMEM-Ham's F-12 medium. The circumferential area of each cyst was measured by using the Image J software (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Data are shown as mean $\pm$ SEM. Once normality was assessed with Kolmogorov-Smirnov or Shapiro-Wilks tests, we used the Student *t* test for statistical comparisons between two groups of normally distributed variables, and one-way analysis of variance (ANOVA) and subsequent *post hoc* tests (Bonferroni, DMS or Tamhane's T2) for comparisons between more than two groups. When non-parametric methods were required, we used the Wilcoxon, Friedman or Kruskal-Wallis and Mann-Whitney tests. Analyses were carried out with GraphPad Prism 5 and/or SPSS statistical packages. Two-tailed *P* values < 0.05 were considered statistically significant.

Results

miR-506 is Overexpressed in the Intrahepatic Bile Ducts of PBC Patients

The expression analysis of miR-506 by qPCR showed 3.4-fold upregulation in PBC liver biopsies compared to normal livers (n=6 individuals in each experimental group) (Fig. 1A). To assess the location of miR-506, *in situ* hybridization experiments were carried out in liver samples of PBC patients and compared with normal and PSC liver samples (Fig. 1B). Most PBC liver sections showed marked miR-506 staining, which specifically located in the cholangiocytes lining the intrahepatic bile ducts, rather than in hepatocytes. In normal liver specimens, no detectable staining, was observed in either hepatocytes or bile ducts. On the other hand, only a minority of the PSC samples used as disease control showed a slight staining within few cholangiocytes.

miR-506 Downregulates AE2 Protein Expression in H69 Cholangiocytes

miR-506 overexpression in PBC livers could be responsible, at least in part, for the previously reported diminished AE2 immunoreactivity in the bile ducts of PBC patients.¹⁵ We therefore assessed whether miR-506 could downregulate AE2 protein expression by using the SV40-immortalized normal human cholangiocytes H69 transfected with pre-miR-506 (a miR-506 precursor). Real-time qPCR confirmed that H69 cells transfected with pre-miR-506 for 48 hours overexpressed the mature miR-506 compared with control H69 cholangiocytes transfected with either a pre-miRNA negative or vehicle (Fig. 2A). Noticeably, immunoblot analysis indicated that overexpression of miR-506 in H69 cholangiocytes result in a marked decrease in AE2 protein expression compared to controls (Fig. 2B). At the studied time point, the levels of AE2 mRNA remained unchanged in those cells overexpressing miR-506 (data not shown), and therefore miR-506 appears to modulate AE2 protein expression through sequestration of the AE2 transcript.

miR-506 Binds to AE2 mRNA and Inhibits Protein Translation in H69 Cholangiocytes

To prove that miR-506 may indeed bind its target site in the 3'UTR region of AE2 mRNA and prevent protein translation, we performed additional experiments of luciferase assay and site-directed mutagenesis. H69 cholangiocytes were cotransfected with the CMV-driven luciferase construct Luc-AE2-3'UTR (which contains the wildtype sequence of human AE2-3'UTR mRNA with the predicted miR-506 target) and either pre-miR-506, pre-miRNA negative control or vehicle. As shown in Fig. 3, the luciferase activity of the wildtype construct Luc-AE2-3'UTR was significantly inhibited in cells overexpressing miR-506 compared to cells receiving pre-miRNA negative control (25.45% inhibition) or vehicle (35.04%). On the other hand, the luciferase activity of the wildtype construct Luc-AE2-3'UTR was significantly increased in cells overexpressing anti-miR-506 oligonucleotides compared to cells receiving pre-miRNA negative control or vehicle (49.13% and 41.28% increase, respectively). Site-directed mutagenesis of the putative miR-506 binding site (construct Luc-mut-AE2-3'UTR) prevented the inhibitory effect of pre-miR-506 cotransfection and the stimulatory effect of the cotransfection with anti-miR-506 oligonucleotides (Fig. 3). These data indicate that miR-506 can specifically bind to its predicted target site in the AE2-3'UTR mRNA region to inhibit protein translation.

miR-506 Downregulates AE2-mediated $\text{Cl}^-/\text{HCO}_3^-$ Exchange Activity in H69 Cholangiocytes

We previously showed that secretion of bicarbonate through $\text{Cl}^-/\text{HCO}_3^-$ exchange activity is only mediated by AE2 in human cholangiocytes.¹² Here, we extended our studies to the functional level and assessed whether the decrease in AE2 protein elicited by miR-506 overexpression could result in diminished anion exchange activity. Cultured H69

cholangiocytes transfected with pre-miR-506 for 48 hours (which are therefore overexpressing miR-506) were trypsinized and seeded for microfluorimetric monitoring of pH_i variations upon perfusion maneuvers, i.e. pH_i alkalization upon Cl^- removal (which forces AE2 to operate in a reverse mode, extruding intracellular Cl^- in exchange with HCO_3^-) and further normalization of the pH_i after restoration of extracellular Cl^- (which allows AE2 to operate in a physiological mode secreting HCO_3^-). As illustrated in Fig. 4A and B, the rates of initial alkalization and subsequent pH_i recovery obtained by these maneuvers were both markedly decreased in H69 cholangiocytes transfected with pre-miR-506 vs control transfected cholangiocytes. These data indicate that overexpression of miR-506 leads to decreased AE2 activity in human cholangiocytes.

miR-506 Overexpression Inhibits the Apical Hydroionic Fluxes Stimulated by Secretin in 3D-Cultured H69 Cholangiocytes

Also, we investigated the effect of miR-506 on the hydrocholeretic function of human cholangiocytes using the model of 3D-cultured H69 cholangiocytes. Under three-dimensional conditions, H69 human cholangiocytes may form cystic structures which spontaneously expand over time as a consequence of fluid secretion into the cyst lumen. As previously reported for 3D-cystic structures derived from rat cholangiocytes,³² the expansion rate of the human H69 cystic structures was accelerated by the presence of secretin in the culture medium during 30 minutes ($6.40 \pm 1.26\%$; $P = 0.0002$; Fig. 5), indicating an increase in fluid secretion to the cyst lumen. But preincubation with pre-miRNA-506 in the culture medium for 24 hours blocked the secretin-stimulated expansion of H69 cholangiocyte cystic structures. Altogether, our findings indicate that upregulated miR-506 may inhibit both AE2 expression and AE2-mediated hydrocholeretic function in human cholangiocytes.

Decreased AE2 Activity in PBC Cholangiocytes May Be Caused by miR-506 Overexpression

Next, we investigated whether our previous data of decreased AE2 activity in cultured PBC cholangiocytes¹⁶ could be related to overexpression of miR-506. We isolated PBC cholangiocytes using pieces of a liver explant from a female patient with PBC and cultured them for 7–10 passages. Also we isolated normal cholangiocytes from liver pieces of normal bordering tissue (obtained during a surgical intervention in a female patient; cf. Supplementary Information) and cultured them as for PBC cholangiocytes. qPCR analysis of miR-506 levels revealed a 2-fold increase in cultured PBC cholangiocytes vs normal cholangiocytes (Fig. 6A). However, the expression levels of two other miRNAs predicted to potentially target human AE2 mRNA, i.e. miR-149-3p and miR-765, were downregulated in cultured PBC cholangiocytes compared to normal cholangiocytes (cf. Supporting Information and Supplementary Fig. 2).

Microfluorimetric studies indicated that miR-506 overexpression in PBC cholangiocytes was associated with decreased AE2 activity (Fig. 6B). To test the hypothesis that the downregulated AE2 activity in PBC cholangiocytes is at least partially due to the increased miR-506 levels, we used commercial anti-miR-506 oligonucleotides to inhibit miR-506. As shown in Fig. 6B, miR-506 inhibition resulted in significantly increased AE2 activity compared with the activity of PBC cholangiocytes receiving pre-miRNA negative control or vehicle. These results were also associated with consistent changes in the AE2 protein expression (Fig. 6C).

Discussion

The key findings reported here relate to the cellular mechanisms accounting for AE2 downregulation in the biliary epithelium of PBC patients. We identified miRNA-506 as a candidate to modulate AE2 and carried out experiments to determine its actual role for AE2 expression in cholangiocytes. Our data indicate that: *i*) miR-506 is overexpressed in the liver of PBC patients compared to normal controls (as demonstrated by qPCR); *ii*) miR-506 overexpression takes place in the intrahepatic bile ducts of PBC patients (as shown by *in situ* hybridization); *iii*) miR-506 is able to bind specifically to the 3'UTR region of AE2 mRNA preventing protein expression (as shown by luciferase-reporter assays); *iv*) overexpression of miR-506 in a cholangiocyte cell line leads to a decrease in both AE2 protein expression and anion exchange activity (as demonstrated by immunoblotting and microfluorimetry, respectively); *v*) miR-506 is involved in the diminished AE2 activity observed in cultured PBC cholangiocytes that overexpress this microRNA (as indicated by the partial improvement of the exchange activity upon miR-506 inhibition) and *vi*) an increase in AE2 protein is induced in these cells after treatment with anti-miR-506. Our data are consistent with the notion that miR-506 may control AE2 expression in cholangiocytes and play an important role in the pathogenesis of PBC.

PBC is a disease with an obscure etiopathogenesis in which intralobular bile ducts are selectively damaged by autoreactive T cells.^{1–4} We had previously reported that AE2 expression is decreased in the liver and peripheral blood mononuclear cells from PBC patients.^{15,34} Moreover, the cAMP-stimulated $\text{Cl}^-/\text{HCO}_3^-$ exchange activity, which in human cholangiocytes is only mediated by AE2,¹² was found to be diminished in cultured PBC cholangiocytes.¹⁶ Our recent findings that *Ae2_{a,b}*-deficient mice develop biochemical, histological, and immunologic alterations that recapitulate many of the features of PBC indeed support the hypothesis that AE2 dysfunctions may have an important role in the pathogenesis of the disease.²¹ It is quite possible that AE2 deficiency in PBC patients may render cholangiocytes more immunogenic and susceptible to autoimmune attack while an equivalent defect in lymphocytes may alter immunological homeostasis leading to autoimmunity.³⁵ However, why AE2 expression and activity are downregulated in bile ducts from PBC patients is unknown.

miRNAs are recognized as important regulators of cell function.^{22–24} Recently, microarray-scan studies in liver tissue identified several differentially expressed miRNAs in PBC.²⁶ Using *in silico* approaches we found that one of the miRNAs which were upregulated in PBC livers,²⁶ miR-506, exhibits base complementarities to the 3'UTR region of AE2 mRNA and could possibly target this messenger. Thus, miR-506 was a prime candidate to potentially account for the downregulation of AE2. The expression analyses of miR-506 by qPCR revealed over 3-fold upregulation in PBC liver biopsies compared to normal liver specimens (Fig. 1A). Moreover, *in situ* hybridization showed that miR-506 overexpression in PBC is mainly located in cholangiocytes of intrahepatic bile ducts (Fig. 1B). No detectable staining was observed in normal tissue specimens and only very limited miR-506 expression was found in PSC samples, suggesting that overexpression of miR-506 is characteristic of PBC. In fact, miR-506 overexpression could also be recognized in freshly isolated and cultured PBC cholangiocytes, which were confirmed to have decreased AE2 activity as previously reported.¹⁶ Of interest, the cause-effect relationship between miR-506 overexpression and decreased AE2 activity in PBC cholangiocytes was hereby substantiated by our finding that blockage of miR-506 with anti-miR-506 oligonucleotides could partially recover the diminished AE2 expression and activity in PBC cholangiocytes.

Experiments of luciferase assay and site-directed mutagenesis in the human cholangiocyte cell line H69 showed that miR-506 may specifically bind its target site in the AE2 mRNA 3'

region and prevent protein translation. Moreover, we extended our studies in this cell line to the functional level and ascertained that downregulation of AE2 protein expression by miR-506 leads to decreased AE2 anion exchange activity. Moreover, we used the model of 3D-cultured H69 cholangiocytes to investigate the effect of miR-506 on the hydrocholeretic function of human cholangiocytes. Under 3D conditions, H69 cholangiocytes formed cystic structures which accelerated their spontaneous expansion upon secretin stimulation because of an increase in fluid secretion to the cyst lumen (similarly to what it was already reported for 3D-cystic structures derived from rat cholangiocytes).³² Interestingly, the presence of pre-miR-506 in the culture medium blocked the secretin-stimulated expansion of H69 cholangiocyte cystic structures. Altogether, our data indicate that overexpression of miR-506 is able to inhibit both AE2 protein expression and AE2-mediated hydrocholeretic function in human cholangiocytes.

Under our experimental conditions, miR-506 appears to modulate AE2 through sequestration rather than degradation of the AE2 message, since H69 cells transfected with pre-miR-506 showed no decrease in the AE2 mRNA levels (data not shown). Concerning the decreased AE2 mRNA expression previously reported in PBC livers,³⁴ it is possible that a chronic upregulation of miR-506 (vs acute) might result in AE2 mRNA degradation. Moreover, other features different from miR-506 upregulation (like gene sequence variations, epigenetic modifications, abnormalities affecting transcription factors, and even diverse miRNAs) might additionally be involved, an issue that needs to be fully clarified in future studies. At this moment we know that other miRNAs predicted to potentially target human AE2 mRNA like miR-149-3p and miR-765 were downregulated in cultured PBC cholangiocytes vs normal cholangiocytes (Supplementary Fig. 2), and that expression of a third candidate, miR-944, was undetectable in both PBC and normal cholangiocytes. Thus, the particular role of miR-506 for the decreased AE2 in PBC livers appears to be further enlightened. While peripheral blood mononuclear cells were also reported to have decreased AE2 mRNA expression in PBC,³⁴ this decreased transcriptional expression is however unrelated to miR-506, as we found no upregulation of miR-506 in the peripheral blood cells collected from patients with PBC (data not shown).

PBC is currently regarded as a multifactorial liver disease that may ensue from highly complex interactions of genetic and environmental-related factors, a view that is being stressed by recent results of genome-wide association studies (GWAS)^{36–39} and more conventional genetic and epidemiological studies.^{40–43} Exposure of susceptible individuals to environmental agents like chemical/xenobiotics, tobacco, alcohol, and a variety of microorganisms may result in epigenetic alterations, which might include not only DNA methylation and histone modification but also dysregulated expression of miRNAs.²³ Indeed, miRNAs are recently being considered as authentic effectors of environmental influences on gene expression and disease.²³ The mechanisms leading to miR-506 upregulation in PBC cholangiocytes remain to be elucidated, and involvement of environmental agents may be postulated. Although *in vivo* animal experiments sound attractive to further explore this issue, unfortunately rat and mouse animal models are not suitable, as the AE2 target sequence is present in the human AE2 mRNA²⁸ but not in the orthologous sequences in rats and mice. On the other hand, the consequences of miR-506 upregulation in the biliary epithelium might be more extensive than primarily inferred from its effects on AE2, and this possibility will certainly deserve further investigation in the future. Thus, miR-506 was also predicted by bioinformatic approaches to potentially target the 3'UTR region of human CK19 mRNA, with base complementarities to the sequence UGUCCUUUGGAGGGUGUCUUC. Interestingly, our western blot data indicate that overexpression of miR-506 in H69 cholangiocytes result in downregulation of CK19 protein expression (cf. Supporting Information and Supplementary Fig. 3).

In summary, our results identify a molecular mechanism (i.e. miRNA suppression of protein translation) which can account for the downregulation of AE2 protein and activity in cholangiocytes of patients with PBC and provide direct evidence that a specific miRNA (i.e. miR-506) is important in the process. Our data therefore introduce the concept that miRNA dysfunction may be central to the pathogenesis of PBC. Moreover they indicate that miR-506 is a potential target to restore the AE2 function in PBC cholangiocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

AE2 (or SLC4A2)	Cl ⁻ /HCO ₃ ⁻ anion exchanger 2
AMA	anti-mitochondrial antibodies
LNA-ISH	locked nucleic acid-based – <i>in situ</i> hybridization
miR	microRNA
PBC	primary biliary cirrhosis
pH_i	intracellular pH
pre-miR	miRNA precursor
PSC	primary sclerosing cholangitis
RT-PCR	reverse transcription – polymerase chain reaction
qPCR	real-time quantitative polymerase chain reaction
UDCA	ursodeoxycholic acid

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B **Fig. 1.**

miR-506 is overexpressed in the liver of PBC patients. (A) Real-time qPCR indicate that the levels of miR-506 in whole liver extracts are increased in PBC compared to normal controls (3.4-fold in the mean values, as indicated by bars; dots indicate the values of each case). The inset illustrates an alignment of the miRNA-506 sequence and its target in the 3'UTR region of AE2 mRNA. (B) *In situ* hybridization for miR-506 in liver tissue by using an anti-miR-506 probe: representative image of a PBC liver section with notable miR-506 staining (green) in the intrahepatic bile ducts (which are visualized in red through CK19 staining after using a TRITC-labeled secondary antibodies). Representative images of normal and PSC liver control sections indicating no detectable miR-506 expression are included as well (cf. images to the *left*). The staining detected in PBC cholangiocytes with anti-miR-506 was specific, as no equivalent green staining could be observed in the PBC liver section when incubated with a negative control (scramble) probe (cf. images to the *right*).

A

B

Fig. 2.

AE2 protein expression is downregulated by miR-506. (A) qPCR indicates that H69 human cholangiocytes transfected for 48 hours with a miR-506 precursor (pre-miR-506) have increased expression of miR-506 compared to H69 cells that received either vehicle or the pre-miRNA negative control, pre-miR(-); values are given as fold levels relative to vehicle control levels. (B) Representative immunoblot showing that overexpression of miR-506 in H69 human cholangiocytes leads to downregulation of AE2 protein expression 48 hours after transfection compared to cells receiving vehicle or the pre-miRNA negative control. β -actin was used as a normalizing loading control. Bottom panel shows the quantitation of

AE2 protein levels relative to β -actin expression; values are given as percentage relative to cells receiving just vehicle. n, number of wells analyzed in each group.

Fig. 3. miR-506 targets the 3'UTR region of human AE2 mRNA. Upper inset illustrates the miR-506 target sequences (wildtype and mutated) of the 3'UTR region of human AE2 mRNA which are contained in the respective luciferase reporter constructs (Luc-AE2-3'UTR and Luc-mut-AE2-3'UTR). In cultured H69 human cholangiocytes transfected with the wildtype Luc-AE2-3'UTR recombinant vector, translation of the luciferase reporter (detected as luciferase activity) was significantly decreased when H69 cells were cotransfected with the miR-506 precursor vs cells cotransfected with either a pre-miRNA negative control (~25% inhibition) or just vehicle (~35%). Cells cotransfected with Luc-AE2-3'UTR and anti-miR-506 oligonucleotides showed increased luciferase activity vs both

cells receiving pre-miRNA negative control (~50% increase) or vehicle (~40%). Site-directed mutagenesis of the predicted miR-506 binding sequence in the Luc-mut-AE2-3'UTR construct eliminated the inhibitory effect of miR-506, as well as the stimulatory effect of anti-miR-506 oligonucleotides, and the luciferase activity remained similar to that observed with vehicle or the pre-miRNA negative control. n, number of wells analyzed in each group.

Fig. 4. miR-506 downregulates the AE2-mediated Cl⁻/HCO₃⁻ exchange activity in H69 human cholangiocytes. (A) Representative traces showing that removal of extracellular Cl⁻ (by perfusion with an isethionate-based medium) leads to intracellular alkalinization as a result of HCO₃⁻ uptake in exchange with Cl⁻ (through an AE2 activity operating in a reverse mode). Restoration of extracellular Cl⁻ decreases the pHi as a result of HCO₃⁻ secretion in exchange with Cl⁻ (physiological AE2 activity). The rates of intracellular alkalinization and subsequent pHi recovery elicited by these maneuvers were downregulated in cultured H69 human cholangiocytes in the presence of the miR-506 precursor (middle panel) vs cells receiving vehicle or a pre-miRNA negative control. (B) Quantitation of the physiological

$\text{Cl}^-/\text{HCO}_3^-$ exchange activity (calculated from the tangent of the experimental plot of intracellular acidification upon restoration of extracellular Cl^- , and expressed as transmembrane base fluxes J_{OH^-}) shows that overexpression of miR-506 (in cells receiving miR-506 precursor) leads to a significant decrease in the AE2 activity compared with cells receiving vehicle or the pre-miRNA negative control. n, number of cells analyzed from 3 independent experiments for each condition.

Fig. 5. miR-506 inhibits the apical hydroionic fluxes stimulated by secretin in 3D-cultured H69 cholangiocytes. Secretin-stimulated expansion of the cystic structures formed by 3D-cultured H69 cells was significantly inhibited when cystic structures had been preincubated for 24 hours with pre-miR-506 compared to H69 cystic structures receiving vehicle or the pre-miRNA negative control. n, number of cystic structures analyzed from two independent experiments for each condition.

A

B

C

Fig. 6. Cultured PBC cholangiocytes show decreased AE2 activity which is at least partially due to miR-506 overexpression. (A) qPCR indicated that isolated and cultured PBC cholangiocytes have 2-fold increased miR-506 expression compared to similarly isolated and cultured normal human cholangiocytes; n, number of wells analyzed. (B) Cultured PBC cholangiocytes (*left* black box) show decreased AE2 anion exchange activity compared to normal cholangiocytes (open box). Diminished AE2 activity in PBC cholangiocytes is partially but significantly recovered through overexpression of anti-miRNA-506 oligonucleotides (*right* black box), vs PBC cholangiocytes with either vehicle or the pre-miRNA negative control; n, number of cells analyzed from two independent experiments for

each condition. (C) Immunofluorescent confocal images confirm that cultured PBC cholangiocytes have decreased AE2 protein expression – visualized as green fluorescence after using FITC-labeled secondary antibodies – compared to normal cholangiocytes. PBC cholangiocytes transfected with Cy3-labeled (ref. AM1632, Ambion) anti-miR-506 oligonucleotides (visualized as red dots) show increased AE2 protein expression compared to PBC cholangiocytes transfected with Cy3-labeled pre-miR negative control (also visualized as red dots) or vehicle; nuclei are stained in blue with Topro-3.