Research Article

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miR-425 regulates inflammatory cytokine production in CD4$^+$ T cells via N-Ras upregulation in primary biliary cholangitis

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Background & Aims: Primary biliary cholangitis (PBC) is an autoimmune liver disease of unknown pathogenesis. Consequently, therapeutic targets for PBC have yet to be identified. CD4$^+$ T cells play a pivotal role in immunological dysfunction observed in PBC, and therefore, microRNA (miRNA) and mRNA expression were analysed in CD4$^+$ T cells, to investigate PBC pathogenesis and identify novel therapeutic targets.

Methods: Integral miRNA and mRNA analysis of 14 PBC patients and ten healthy controls was carried out using microarray and quantitative real-time polymerase chain reaction (qRT-PCR), with gene set enrichment analysis. The functional analyses of miRNA were then assessed using reporter and miRNA-overexpression assays.

Results: The integral analysis of miRNA and mRNA identified four significantly downregulated miRNAs (miR-181a, -181b, -374b, and -425) related to the T cell receptor (TCR) signalling pathway in CD4$^+$ T cells of PBC. *N-Ras*, a regulator of the TCR signalling pathway, was found to be targeted by all four identified miRNAs. In addition, *in vitro* assays confirmed that decreased miR-425 strongly induced inflammatory cytokines (interleukin [IL]-2 and interferon [IFN]-γ) via N-Ras upregulation in the TCR signalling pathway.

Conclusion: The decreased expression of four miRNAs that dysregulate TCR signalling in PBC CD4$^+$ T cells was identified. miR-425 was demonstrated as an inflammatory regulator of PBC via N-Ras upregulation. Therefore, the restoration of decreased miR-425 or the suppression of N-Ras may be a promising immunotherapeutic strategy against PBC.

Lay summary: Primary biliary cholangitis (PBC) is an autoimmune liver disease, but the causes are unknown. MicroRNAs are molecules known to regulate biological signals. In this study, four microRNAs were identified as being decreased in PBC patients, leading to activation of T cell receptor signalling pathways, involved in inflammation. One particular target, N-Ras, could be an attractive and novel immunotherapeutic option for PBC.

Transcript profiling: Microarray data are deposited in GEO (GEO accession: GSE93172).

Introduction

Primary biliary cholangitis (PBC), formerly known as primary biliary cirrhosis, is a progressive autoimmune liver disease of unknown pathogenesis that predominantly affects middle-aged females [1–3]. Ursodeoxycholic acid (UDCA) treatment slows disease progression in most patients; nevertheless, 10 years after diagnosis, approximately 32% of PBC patients in a late histologic stage and approximately 6% of patients in an early histologic stage, progress to liver transplantation or death due to liver failure [4]. Therefore, there is a substantial clinical need for novel treatments based on an understanding of PBC pathogenesis.

CD4$^+$ T cells play a pivotal role in PBC pathogenesis [5]. Pathologically, the numbers of autoreactive CD4$^+$ T cells against E2 subunits of the pyruvate dehydrogenase complex, the target of anti-mitochondrial antibodies, increase in the peripheral blood, liver and liver-draining lymph nodes of PBC patients [6]. Antigens are presented to CD4$^+$ T cells on the surface of biliary epithelial cells in PBC patients by major histocompatibility complex (MHC)-I and -II proteins [7,8]. Increased levels of interferon (IFN)-γ, a Th1 cytokine, are considered to be a characteristic of PBC [9,10]. However, details of the molecular mechanisms occurring in CD4$^+$ T cells during PBC pathogenesis remain poorly understood.

miRNAs represent a subclass of small noncoding RNAs of 19–24 nucleotides in length that regulate the transcription and translation of target genes via complementary binding to the 3′-untranslated region (UTR) [11–13]. miRNAs target and regulate cell type-specific biological processes, including those of the immune system [14,15]. Recent studies have demonstrated that miRNAs play a critical role in the pathogenesis of various

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liver and autoimmune diseases [16–18]. In PBC, differentially expressed miRNAs have been identified in liver tissue, biliary epithelium, serum, and peripheral blood mononuclear cells (PBMCs) [19–22]. In addition, mRNA profiling studies in PBC have also been reported [7,23,24]. However, no studies have yet analysed miRNA or mRNA expression profiles in $CD4^+$ T cells isolated from PBC patients. Therefore, we aimed to analyse miRNA and mRNA expression integrally in $CD4^+$ T cells isolated from PBC patients to further understand the pathological mechanisms underlying PBC pathogenesis and to identify novel therapeutic targets for PBC. In this study, miRNA and mRNA expression in $CD4^+$ T cells of PBC patients were integrally profiled, miRNA-target gene pairs potentially involved in PBC pathogenesis were elucidated, and dysregulated PBC-associated signalling pathways were identified by uncovering miRNA-target gene pairs in PBC $CD4^+$ T cells. In addition, the regulation of inflammatory cytokine production by identifying miRNA-target gene pairs via the modulation of signalling pathways were also confirmed. The results of the present study demonstrate the identification of miRNAs and corresponding target genes that play an important role in PBC pathogenesis.

Patients and methods

Patients and subjects

All PBC patients were diagnosed according to the clinical criteria (including histology) at an early clinical stage and were receiving treatment with 600 mg/day of UDCA [25]. Healthy controls were not treated with any medicine and had no evidence of liver disease. Total RNA from peripheral $CD4^+$ T cells of patients was assessed by microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR), was carried out as described in the Supplementary materials section.

miRNA-mRNA integral analysis

SurePrint G3 human miRNA microarray kits 8x60K Rel.16.0 (Agilent Technologies, Santa Clara, CA, USA) and SurePrint G3 human GE 8x60K microarrays (Agilent Technologies) were used to analyse the miRNA and mRNA expression profiles for an integral analysis. The expressions of identified miRNA and mRNA were validated by qRT-PCR. Target genes of validated miRNA were predicted using bioinformatics [26–28]. Integral analysis was performed using a gene set enrichment analysis (GSEA), with miRNA-target gene sets as described in the Supplementary materials [29]. Pathway analysis was performed using database annotation visualisation and integrated discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov) and the database of Kyoto encyclopaedia of genes and genomes (KEGG) pathway mapping [30,31].

Table 1. Clinical data of primary biliary cholangitis (PBC) patients and healthy controls.

	PBC (n = 14) Mean, SD	Control (n = 10) Mean, SD	Normal ranges
Age (years)	60.9, 7.2	55.1, 7.6	n.a.
AST (IU/L)	31.8, 14.2	20.4, 2.5*	10–33
ALT (IU/L)	20.6, 6.6	16.7, 4.1	6–35
γ-GT (IU/L)	57.6, 47.2	20.4, 7.2*	9–27
ALP (IU/L)	331, 172.1	188.3, 15.0*	96–300
T-Bil (mg/dl)	0.9, 0.4	n.a.	0.2–1.3
Alb (g/dl)	4.0, 0.5	n.a.	3.5–5.2
PT (%)	88.5, 12.1	100.0, 0.0*	>70
TC (mg/dl)	193.6, 35.9	201.1, 52.8	120–219
M2 (U/ml)	135.0, 70.5	n.a.	<7.0
IgM (mg/dl)	208.2, 132.1	n.a.	35–220

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; T-Bil, total bilirubin; Alb, albumin; PT, prothrombin time; TC, total cholesterol; M2, anti-mitochondrial M2 antibody; IgM, immunoglobulin M; N.A., not available.
* p <0.05.

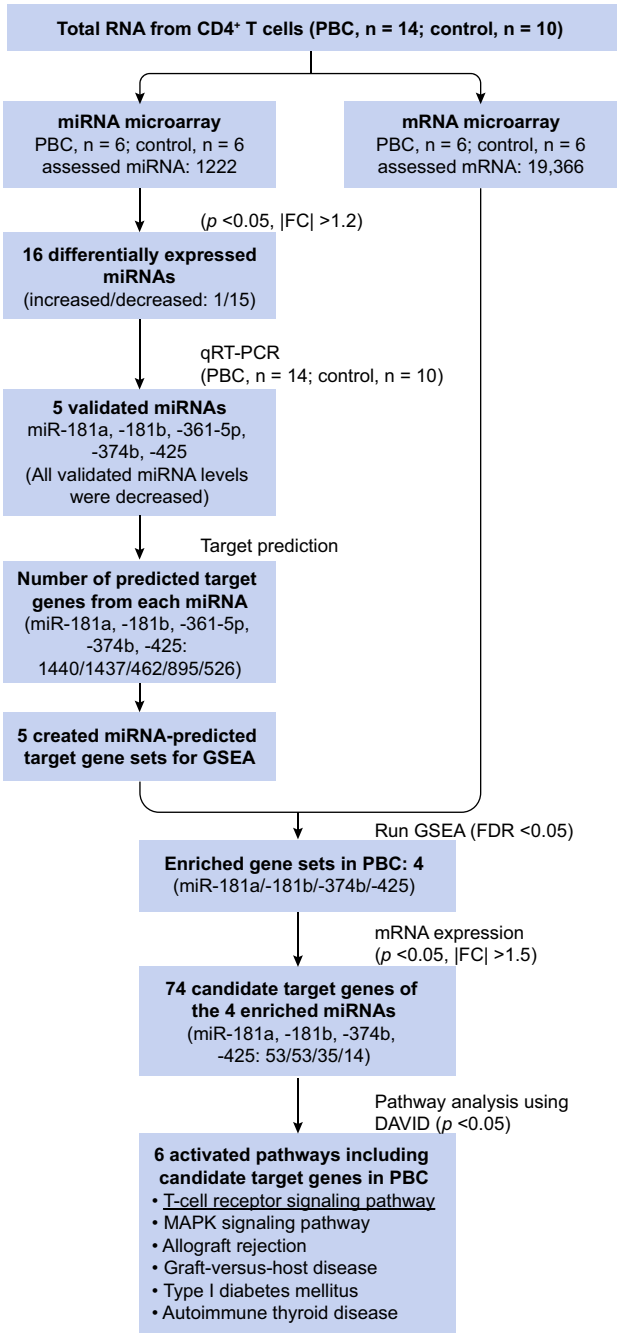

Fig. 1. Schematic of miRNA and mRNA integral analysis of primary biliary cholangitis (PBC) $CD4^+$ T cells. miRNA, microRNA; qRT-PCR, quantitative real-time polymerase chain reaction; FC, fold change; FDR, false discovery rate; GSEA, gene set enrichment analysis; DAVID, database for annotation visualisation and integrated discovery.

In vitro assay

Jurkat cells and HEK293T cells were used for *in vitro* assays. A luciferase assay with a miRNA mimic or locked nucleic acid (LNA) inhibitor was performed to identify miRNA targets. Transfection of plasmid vector and infection of lentiviral vector were performed for functional analysis of miRNA and target genes. An anti-CD3 antibody was used for T cell receptor (TCR) stimulation of cultured cells. Farnesylthiosalicylic acid (FTS) was used for Ras inhibition assay. Each phenotype of treated cells was examined by qRT-PCR, Western blot, and enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

Data are shown as mean with standard deviation (SD) from the mean. The data of two groups were analysed with Student's *t* test. The data of groups greater than or equal to three were analysed by Dunnett's test or Tukey's multiple comparisons test. The value of $p < 0.05$ was considered statistically significant.

Results

miRNA profiles of CD4⁺ T cells from primary biliary cholangitis patients

A total of 14 PBC patients and ten healthy controls (Table 1) were assessed by the following studies (Fig. 1). All of the 14 PBC patients were anti-mitochondrial antibody positive as shown by a chemiluminescence enzyme immunoassay using Stacia MEBLux test mitochondria M2 (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan).

In the miRNA microarray study, total RNAs of CD4⁺ T cells from six PBC patients and six healthy controls were examined. All samples fulfilled the following criteria (RNA integrity number >8.0, A260/A280 ratio; 1.9–2.2). The expression levels of 16 miRNAs were found to differ significantly between PBC patients and controls ($p < 0.05$, fold change >1.2) (Table S1). Of the 16 differentially expressed miRNAs, 15 were downregulated and one was upregulated in PBC patients compared with those in the control group (Fig. 2A and B). In the validation study using qRT-PCR, total RNA of CD4⁺ T cells from 14 PBC patients and ten healthy controls (including the subjects assessed by microarray study) was examined. Of the 16 differentially expressed miRNAs on microarray analysis, five miRNAs (miR-181a, -181b, -361-5p, -374b, and -425) were validated by qRT-PCR to be significantly downregulated in PBC patients, compared with those in the controls (Fig. 2C).

Enrichment analysis for miRNA and target genes

To identify the miRNA-target gene groups potentially involved in PBC pathogenesis, a GSEA was performed. The numbers of predicted target genes for miR-181a, -181b, -361-5p, -374b, and -425 were 1,440, 1,437, 462, 895, and 526, respectively. Then, five miRNA-predicted target gene sets were created by combining each miRNA with corresponding predicted target genes. In mRNA microarray studies for GSEA, 27,958 Entrez genes in CD4⁺ T cells obtained from six PBC patients and six healthy controls were assessed. Of the five validated miRNA-predicted target gene sets, four miRNA-predicted target gene sets (miR-181a, -181b, -374b, and -425) were found to be significantly enriched in PBC patients (false discovery rate [FDR] <0.05; Table 2). From the four enriched miRNA-predicted target gene sets, 74 candidate target genes were extracted from predicted target genes ($p < 0.05$; fold change >1.5). The numbers of candidate target genes for each gene set of

Table 2. Extraction of candidate target genes using GSEA with created gene sets.

Gene set	FDR	Predicted target genes (n)	Candidate target genes (n)
miR-181a	<0.01	1,440	53
miR-181b	<0.01	1,437	53
miR-374b	<0.01	895	35
miR-425	<0.05	526	14

Each miRNA-target gene set was composed of miRNA and the corresponding predicted target genes. GSEA-identified enriched target genes negatively correlated with the four downregulated miRNAs (FDR <0.05). The expression of candidate target genes significantly differed between primary biliary cholangitis (PBC) patients (n = 6) and healthy controls (n = 6) ($p < 0.05$; fold change >1.5). GSEA, gene set enrichment analysis; FDR, false discovery rate.

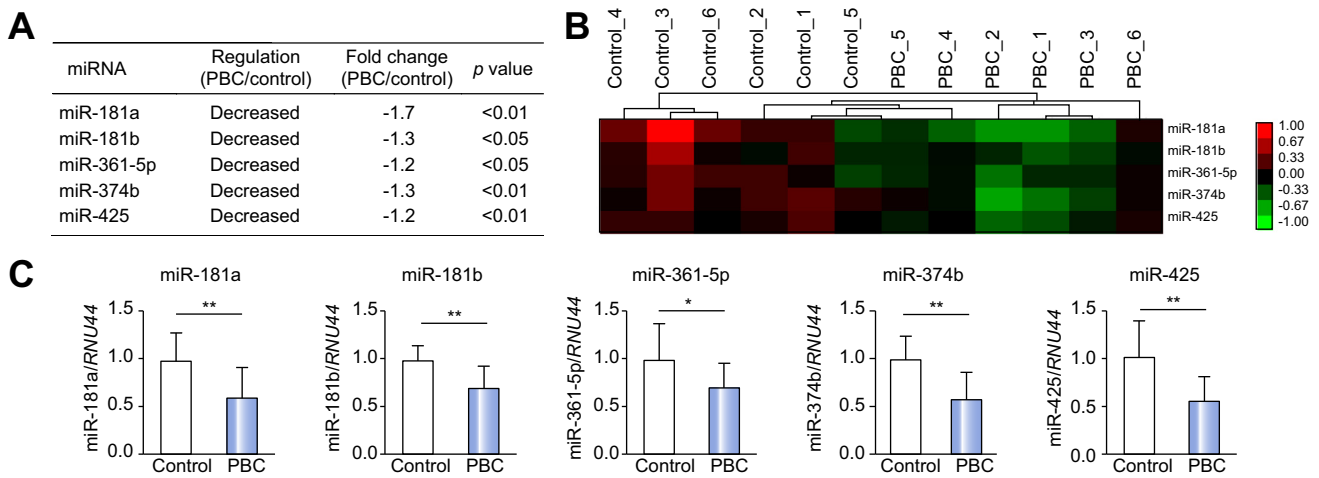

Fig. 2. Profiles of miRNAs in CD4⁺ T cells from PBC patients (n = 6) compared with those from healthy controls (n = 6). (A) The table showed the expression pattern, fold change, and *p* value of five differentially expressed miRNAs ($p < 0.05$, absolute fold change >1.2). (B) The heat map of five differentially expressed miRNAs was created by cluster 3.0 and TreeView 3.0. (C) For all 16 miRNAs, qRT-PCR was performed to validate differential expression between PBC (n = 14) and control (n = 10) subjects ($p < 0.05$). Data in Fig. 2C are expressed as the means of subjects with SD. *p* value in Fig. 2A and Fig. 2C was calculated by Student's *t* test. $^{*}p < 0.05$; $^{**}p < 0.01$.

miR-181a, -181b, -374b, and -425 were 53, 53, 35, and 14 (including overlapping genes), respectively (Table 2).

Pathway analysis of candidate target genes

From the 74 candidate target genes, six pathways (TCR signalling pathway, MAPK signalling pathway, allograft rejection, graft-versus-host disease (GVHD), type I diabetes mellitus and autoimmune thyroid disease) were identified from KEGG pathway analysis by DAVID (p <0.05; Fig. 3A). In total, four identified miRNAs and five candidate target genes (*IFN-γ*, *IL-2*, *IL-10*, *N-RAS*, and *MAP3K8*) were mapped onto the TCR signalling pathway using VisANT ver.1 (http://visant.bu.edu) (Fig. 3B) [32]. The expression levels from all subjects of five candidate target genes on the TCR signalling pathway in $CD4^+$ T cells were validated by qRT-PCR (p <0.05) (Fig. 3C).

A

Pathway	p value	Number of candidate target genes/total number of genes	Candidate target genes
T cell receptor signaling pathway	<0.01	5/108	*NRAS, MAP3K8, IFN-γ, IL-10, IL-2*
MAPK signaling pathway	<0.01	6/267	*DUSP5, NRAS, MAP3K8, DUSP10, FAS, TRAF6*
Allograft rejection	<0.01	4/36	*IFN-γ, FAS, IL-10, IL-2*
Graft-versus-host disease	<0.05	3/39	*IFN-γ, FAS, IL-2*
Type I diabetes mellitus	<0.05	3/42	*IFN-γ, FAS, IL-2*
Autoimmune thyroid disease	<0.05	3/51	*FAS, IL-10, IL-2*

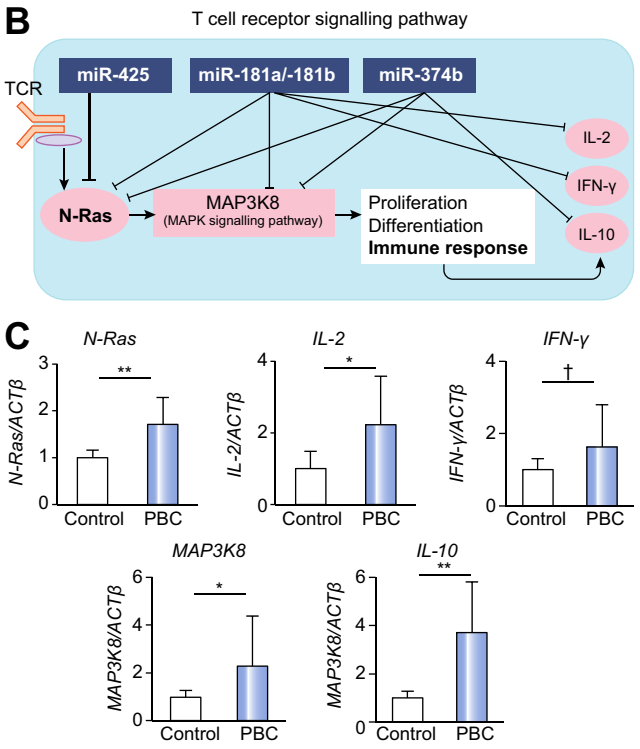

Fig. 3. Pathway analysis of four differentially expressed miRNAs and 74 candidate target genes. (A) Activated pathways with candidate target genes were determined using DAVID (p <0.05). (B) A representation of the T cell receptor signalling pathway was visualised with the four identified miRNAs and five candidate target genes using VisANT ver.1. (C) For all five candidate target genes, qRT-PCR was performed to validate the differential expression between PBC (n = 14) and control (n = 10) subjects. Data in Fig. 3C is expressed as the means of subjects with SD. p value in Fig. 3C was calculated by Student's t test. *p <0.05; **p <0.01; †p <0.1.

Inflammatory cytokine regulation by N-Ras overexpressing Jurkat cells

Overexpression of N-Ras was confirmed by qRT-PCR and Western blot in Jurkat cells stably transfected with N-Ras expression plasmid (Fig. 4A). Expressions of *IL-2* and *IFN-γ* were found to be significantly increased in N-Ras-overexpressing Jurkat cells following anti-CD3 stimulation compared with those in mock control Jurkat cells by qRT-PCR (p <0.05; Fig. 4B). Supernatant IL-2 was found to be significantly increased in N-Ras-overexpressing Jurkat cells following anti-CD3 stimulation compared with those in mock control Jurkat cells by ELISA (p <0.05; Fig. 4C).

N-Ras regulation by the four enriched miRNAs

Bioinformatic analysis showed alignments of four enriched miRNAs and their binding sites in the 3′-UTR of *N-Ras* mRNA (Fig. 5A). miR-425 had four binding sites in the 3′-UTR of *N-Ras* mRNA and was the most frequent miRNA among four enriched miRNAs. To examine the binding between the 3′-UTR of *N-Ras* and the four enriched miRNAs, a luciferase reporter assay with miRNA mimics was performed. A miR-425-mimic and a mixture of all four miRNA mimics significantly decreased Luc-*NRas*-3′UTR (which contains the whole sequence of human *N-Ras*-3′-UTR) activity compared with that in the negative control (Fig. 5B). To verify the specificity of the effect of miR-425 on the 3′-UTR region of *N-Ras*, experiments with mutated miR-425 mimic and miR-425 antisense inhibitor were performed. The miR-425 mimic significantly decreased luciferase activity of Luc-*NRas*-3′-UTR vector compared with the negative control and miR-425-mut mimic (p <0.05, Fig. 5C). On the other hand, miR-425-mut mimic did not change luciferase activity of Luc-*NRas*-3′-UTR vector compared with the negative control (Fig. 5C). Moreover, the miR-425-inhibitor restored this decrease of luciferase activity of Luc-*NRas*-3′UTR by the miR-425-mimic (p <0.01, Fig. 5C). To verify the specificity of the effect of miR-425 on the miR-425 binding site of *N-Ras*, experiments with Luc-*NRas*-miR425 binding site vector (which contains tandemly lined up miR-425 binding site sequences of human *N-Ras* 3′-UTR) and Luc-mut-*NRas*-miR425 binding site vector (which contains tandemly lined up mutated miR-425 binding site sequences of human *N-Ras* 3′-UTR) were performed. The luciferase activity of Luc-*NRas*-miR425 binding site vector was significantly decreased by miR-425 mimic compared with the negative control mimic (p <0.05, Fig. 5D). On the other hand, luciferase activity of Luc-mut-*NRas*-miR425 binding site vector was abrogated the decreasing effect of miR-425 mimic (Fig. 5D).

A high expression of miR-425 was confirmed by qRT-PCR in Jurkat cells stably infected with miR-425 lentiviral vector (Fig. 5E). N-Ras mRNA and protein levels were measured in miR-425-overexpressing Jurkat cells by qRT-PCR and Western blot. The expression levels of *N-Ras* mRNA were significantly decreased in miR-425-overexpressing Jurkat cells compared with those in the control (p <0.05; Fig. 5F). Likewise, N-Ras protein

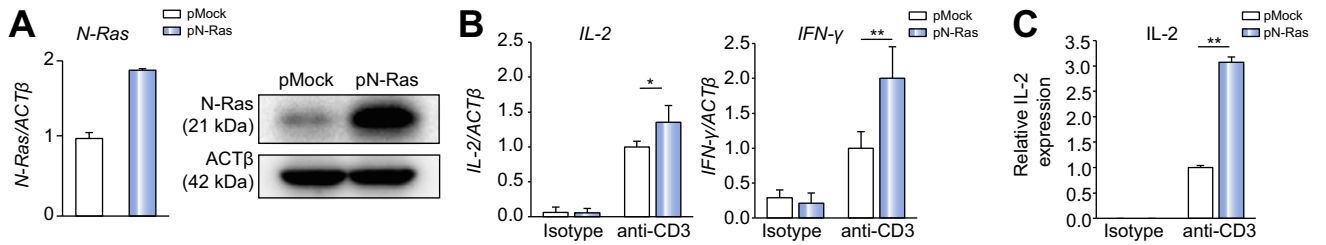

Fig. 4. N-Ras overexpression in Jurkat cells. (A) N-Ras expression of transfected Jurkat cells was confirmed by qRT-PCR (left) and Western blot (right) analysis. (B) The expression levels of *IL-2* and *IFN-γ* induced by anti-CD3 antibody in N-Ras-overexpressing Jurkat cells and mock controls were assessed by qRT-PCR (n = 3, p <0.05). (C) The IL-2 production in N-Ras-overexpressing Jurkat cells and mock controls was assessed by enzyme-linked immunosorbent assay (ELISA). Data in Fig. 4A–C are expressed as the means of subjects with SD. p value in Fig. 4B, Fig. 4C was calculated by Student's t test. $^{*}p$ <0.05; $^{**}p$ <0.01; pMock, mock vector transfected Jurkat cells; pN-Ras, N-Ras expression vector transfected Jurkat cells.

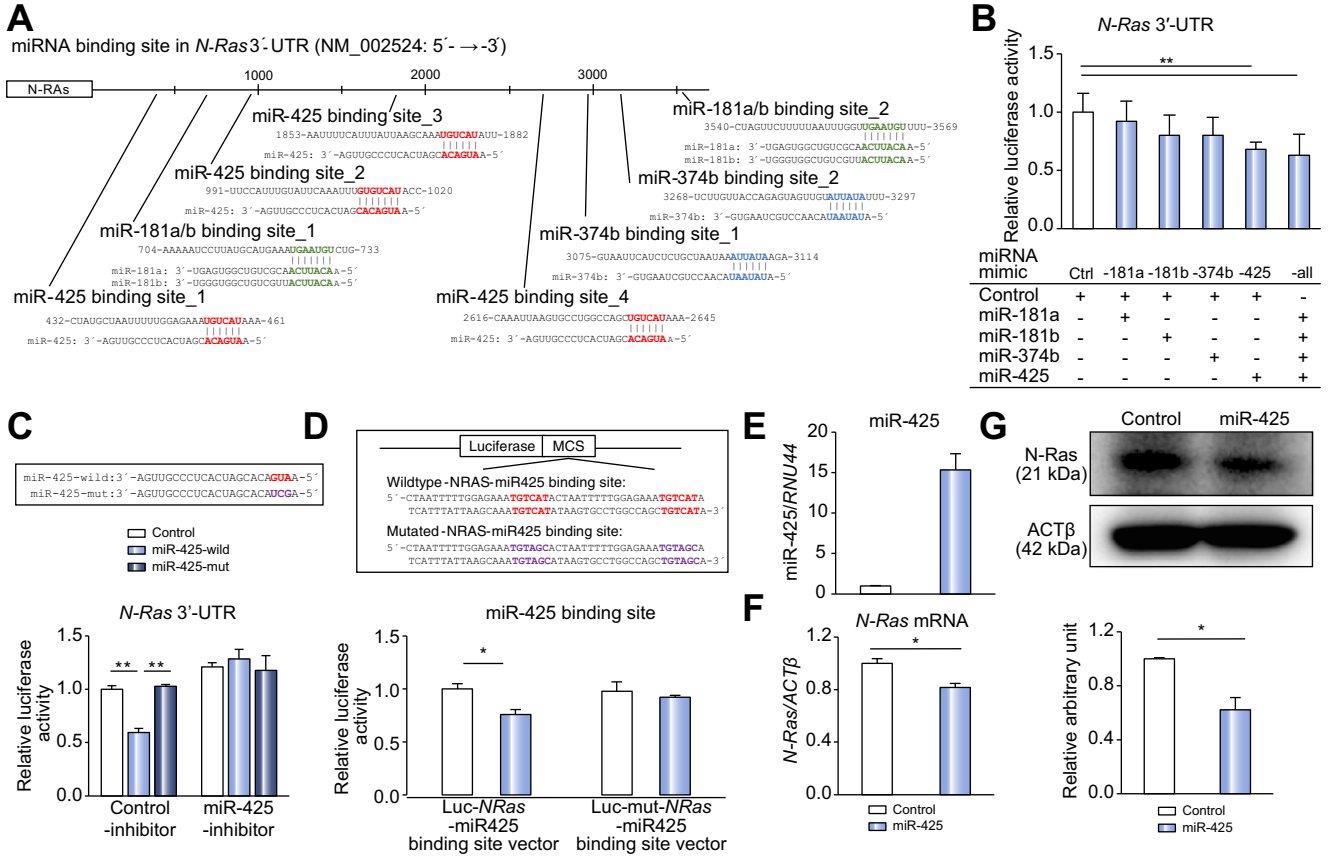

Fig. 5. N-Ras regulation by the four identified miRNAs. (A) Alignments of four enriched miRNAs and their binding sites in the 3′-UTR of *N-Ras* mRNA were derived from the database of Targetscanv.6.2. (B) To examine the binding between *N-Ras* 3′-UTR and the four identified miRNAs, a luciferase reporter assay with the four miRNA mimics was performed (n = 3, p <0.05). (C) To validate the *N-Ras* regulation by miR-425, an *N-Ras* 3′-UTR luciferase reporter assay with miR-425 mimic (wild-type and mutant) or miR-425-inhibitor was performed (n = 3, p <0.05). The top panel shows miR-425 mimic sequences (wild-type and mutant). (D) To prove specificity of the decreasing effect of miR-425 on N-Ras expression, experiments with Luc-*NRas*-miR425 binding site vector and Luc-mut-*NRas*-miR425 binding site vector using miR-425 mimic were performed. The upper panel shows the sequences of wild-type and mutated *NRas*-miR425 binding site. In the bottom panel, miR-425 mimic is shown to inhibit luciferase activity of Luc-*NRas*-miR425 binding site vector significantly compared to the negative control mimic (n = 3, p <0.05). Luciferase activity of Luc-mut-*NRas*-miR425 binding site vector abrogated the inhibitory effect of miR-425 mimic. (E) miR-425 expression of Jurkat cells infected with lentivirus was confirmed by qRT-PCR. The confirmed cells were then used for the following experiments. (F) *N-Ras* mRNA expression in miR-425 overexpressing and mock control cells was assessed by qRT-PCR (n = 3, p <0.05). (G) N-Ras-protein expression in miR-425 overexpressing and mock control cells was assessed by Western blot analysis (n = 3, p <0.05). The top panel shows the representative immunoblotting. The bottom panel indicated values of relative N-ras expression normalised to β-actin expression in miR-425 overexpressing and mock control cells. Data in Fig. 5B-G are expressed as the means of subjects with SD. p value was calculated by Dunnett's test in Fig. 5B, Fig. 5D, Tukey's multiple comparisons test in Fig. 5C, and Student's t test in Fig. 5F, Fig. 5G. $^{*}p$ <0.05; $^{**}p$ <0.01; UTR, untranslated region; MCS, multiple cloning site; Mock, mock lentiviral vector infected Jurkat cells; miR-425, miR-425 expression lentiviral vector infected Jurkat cells; Isotype, stimulated by isotype control; anti-CD3, stimulated by anti-CD3 antibody.

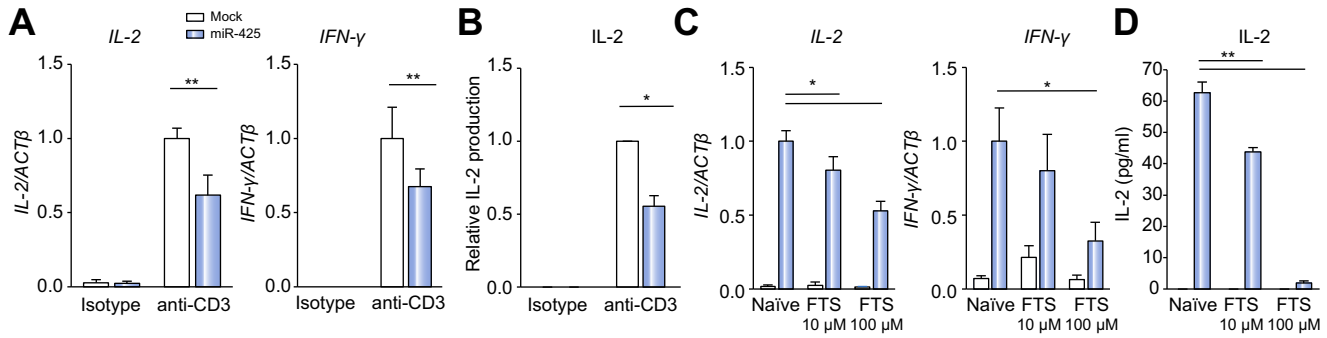

Fig. 6. Cytokine regulation via N-Ras inhibition by miR-425. (A) The expression levels of *IL-2* and *IFN-γ* induced by anti-CD3 antibody in miR-425 overexpressing Jurkat cells and mock controls were assessed by qRT-PCR (n = 3, p <0.05). (B) IL-2 production by the anti-CD3 antibody in miR-425 overexpressing Jurkat cells and mock controls was assessed by ELISA (n = 3, p <0.05). (C) The expression levels of *IL-2* and *IFN-γ* induced by the anti-CD3 antibody with either 10 μM or 100 μM FTS treatment were assessed by qRT-PCR. (D) IL-2 production induced by anti-CD3 antibody with either 10 μM or 100 μM FTS treatment was assessed by ELISA (n = 3, p <0.05). Data in Fig. 6A–D are expressed as the means of subjects with SD. p value was calculated by Student's t test in Fig. 6A, Fig. 6B and Dunnett's test in Fig. 6C, Fig. 6D. $^{*}p$ <0.05; $^{**}p$ <0.01; Mock, mock lentiviral vector infected Jurkat cells; miR-425, miR-425 expression lentiviral vector infected Jurkat cells; Isotype, stimulated by isotype control; anti-CD3, stimulated by anti-CD3 antibody; FTS, farnesylthiosalicylic acid.

levels were significantly decreased in miR-425-overexpressing Jurkat cells compared with those in the control (p <0.05; Fig. 5G).

N-Ras suppression by miR-425 and farnesylthiosalicylic acid

In miR-425-overexpressing Jurkat cells, the expressions of *IL-2* and *IFN-γ* were lower than those in mock control Jurkat cells (Fig. 6A). In fact, supernatant IL-2 levels were found to be significantly decreased in miR-425-overexpressing Jurkat cells following anti-CD3 stimulation compared with those in mock control Jurkat cells (p <0.05; Fig. 6B). FTS significantly suppressed *IL-2* and *IFN-γ* expression in CD3-stimulated Jurkat cells by inhibiting N-Ras, as did miR-425 by suppressing N-Ras (p <0.05, Fig. 6C). Likewise, suppressed IL-2 levels, measured by ELISA, were found to be significant by a decrease in FTS-treated Jurkat cells following anti-CD3 stimulation compared with those in mock control Jurkat cells (p <0.05, Fig. 6D).

Discussion

The results of the present study demonstrated that the decreased expression of four miRNAs (miR-181a, -181b, -374b, and -425) in $CD4^+$ T cells isolated from PBC patients may lead to increased inflammatory cytokine production via the TCR signalling pathway. In particular, miR-425 regulated TCR signalling to induce inflammatory cytokines via N-Ras expression.

Notably, integral miRNA-mRNA analysis using GSEA visualised molecular biological disorders that had occurred in $CD4^+$ T cells of PBC. The individual study of miRNA or mRNA profiling in PBC could not overlook the interactive reactions between miRNA and its target genes [19,21,22]. Therefore, we investigated miRNA and target gene expression in the pathogenesis of PBC by integral analysis. On the other hand, integral analysis requires a relatively large quantity of total RNA, as well as a high concentration and quality of total RNA. Unfortunately, it is currently impossible to perform integral analyses in more specific T cell subsets (e.g. Th1, Th17, and regulatory T cells) as these techniques require greater numbers of cells than can be readily obtained [33,34]. Needless to say, miRNA expression profiles are known to differ between lymphocyte subsets [35,36]. Nevertheless, integral analysis of specific T cell subsets has not yet been reported, and the present study is the first report to analyse integrally and comprehensively miRNA-mRNA profiles in an inflammatory disease.

To demonstrate the pathological role of miRNA and its target genes in $CD4^+$ T cells of PBC, we focused on *N-Ras* in the present study because *N-Ras* was targeted by all four identified miRNAs and was upstream of the TCR signalling pathway. Moreover, N-Ras is known to play a pivotal role in T cell activation and immunological reactions among the Ras family (N-Ras, H-Ras and, K-Ras) [37–40]. Indeed, *N-Ras* was shown to be regulated by miR-425 and affected cytokine production in T cells in the present study. Thus, the restoration of decreased miR-425, leading to N-Ras inhibition, was expected to suppress inflammation in PBC. Recently, Ras inhibitors have become the focus as potential immunosuppressors for inflammatory diseases [41–43]. The present study firstly described PBC as an N-Ras-overexpressed inflammatory disease. Additionally, N-Ras is reportedly a senescence-associated molecule in the cholangiocyte of primary sclerosing cholangitis and PBC [44]. Ras inhibition may improve the disorders of cholangiocytes in PBC. Furthermore, the present study has shown that the GVHD pathway and the allograft rejection pathway were upregulated in $CD4^+$ T cells of PBC compared to those of normal controls. PBC-like cholangitis sometimes occurs in chronic GVHD [45]. Although cholangitis in chronic GVHD is pathologically different from that in PBC [46], our pathway analysis suggested that shared mechanisms underlie both diseases to destruct cholangiocytes by $CD4^+$ T cells. Interestingly, Ras inhibitors are reported to prevent GVHD [47]. Thus, it is possible that Ras inhibitors may also prevent the destruction of cholangiocytes by $CD4^+$ T cells of PBC. Therefore, inhibition of N-Ras seems to be an appropriate treatment option for PBC.

N-Ras inhibition by miR-425 is unique compared to other Ras inhibitors. miR-425 specifically suppressed N-Ras translation among the Ras family because sequences of *H-Ras* and *K-Ras* 3′-UTRs do not contain the miR-425-seed site as strongly. Almost all Ras inhibitors target and suppress prenylations (mainly farnesyl transferase) in the Ras activation cascade and affect the entire Ras family [48]. Thus clinical trials showed that certain side

effects (mainly diarrhoea) occurred in Ras inhibitor recipients [49,50]. Therefore, immunosuppression via N-Ras inhibition by the restoration of decreased miR-425 is expected to reduce side effects of conventional Ras inhibitors. To establish the miR-425 treatment for PBC, we should identify miR-425 inducers in T cells. Reportedly, oestrogen is a miR-425 inducer in breast cancer [51]. Therefore, we are trying to regulate miR-425 expression by certain substrates. Unfortunately, we were unable to induce miR-425 in Jurkat cells by oestrogen treatment (data not shown). Further studies are required to establish a miR-425-dependent therapy for PBC.

Finally, microarray study and target prediction suggest four enriched miRNAs may regulate *N-Ras* concordantly. miR-181a, -181b, and -374b seem to be mild regulators of *N-Ras*. In fact, miR-181a modulates inflammatory cytokine production in T cells [36], and miR-374b has been reported to regulate AKT1, included in the TCR signaling pathway, in T cell lymphoma [52]. Thus, miR-181a, -181b, and -374b possibly regulate inflammatory cytokine production not only via *N-Ras* regulation, but also via other target genes in $CD4^+$ T cell of PBC.

In summary, we identified decreased levels of miRNAs that may cause dysregulated inflammatory cytokine production via the modulation of the TCR signalling pathway in $CD4^+$ T cells isolated from PBC patients. The findings of the present study may contribute to the development of novel therapeutic options for PBC.

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Conflict of interest

Dr. Nakagawa reports grants from JSPS KAKENHI Grant Number 16K19330, during the conduct of the study. Dr. Zeniya reports grants from Diseases, the intractable hepato-biliary disease study group in Japan, during the conduct of the study. Dr. Kato reports grants from The Ministry of Education, Culture, Sports, Science and Technology, Japan, grants from Japan Agency for Medical Research and Development, Japan, during the conduct of the study; grants from Scholarship donation of Bristol-Myers Squibb, grants from Scholarship donation of Gilead Sciences, grants from Scholarship donation of Chugai Pharmaceutical co., ltd., outside the submitted work. The other authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

M.S., N.K. and M.Z. conceived the study. R.N. performed the experiments and analysed the data together with R.M., C.S., Y.K., K.G., I.S., and Y.M. T.K., M.N., and K.K. performed the material support. N.K. co-wrote the paper. All authors discussed the results and commented on the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2017.02.002.

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