Global miRNA profiling reveals key molecules that contribute to different chronic lymphocytic leukemia incidences in Asian and Western populations

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Received: March 21, 2023.
Accepted: August 14, 2023.
Early view: August 31, 2023.

https://doi.org/10.3324/haematol.2023.283181

Abstract

It has been known for decades that the incidence of chronic lymphocytic leukemia (CLL) is significantly lower in Asia than in Western countries, but the reason responsible for this difference still remains a major knowledge gap. Using GeneChip® miRNA array to analyze the global microRNA expression in B lymphocytes from Asian and Western CLL patients and healthy individuals, we have identified microRNA with CLL-promoting or suppressive functions that are differentially expressed in Asian and Western individuals. In particular, miR-4485 is upregulated in CLL patients of both ethnic groups, and its expression is significantly lower in Asian healthy individuals. Genetic silencing of miR-4485 in CLL cells suppresses leukemia cell growth, whereas ectopic expression of miR-4485 promotes cell proliferation. Mechanistically, miR-4485 exerts its CLL-promoting activity by inhibiting the expression of TGR5 and activating the ERK1/2 pathway. In contrast, miR-138, miR-181a, miR-181c, miR-181d, and miR-363 with tumor-suppressive function are highly expressed in Asian healthy individuals. Our study suggests that differential expression of several important microRNA with pro- or anti-CLL functions in Asian and Western B lymphocytes likely contributes to the difference in CLL incidence between the two ethnic groups, and that miR-4485 and its downstream molecule TGR5 could be potential therapeutic targets.

Introduction

Chronic lymphocytic leukemia (CLL) is a malignancy characterized by the accumulation of mature but dysfunctional B lymphocytes with abnormal expression of CD5.[1-3] CLL is the most common form of adult leukemia in the United States and western European countries, but is rare in the Asian population.[4-10] Although the exact reasons for such a substantial difference in disease incidences remain unclear, it appears that genetic rather than environmental factors are the most likely mechanistic explanation. Genetic contribution to CLL incidence was previously suggested by observational studies showing that individuals from Japan who settled in Hawaii did not exhibit any increase in CLL incidence.[11,12] Further evidence supporting the strong genetic impact on CLL incidence was provided by a study which analyzed the Los Angeles County Population-based Cancer Registry and showed that Asians (Chinese, Japanese, Filipinos, and Koreans) had a significantly lower incidence of CLL compared to non-Hispanic whites in the same geographic region.[13] Interestingly, the same study also revealed that the birthplace or socioeconomic status did not account for the

difference in CLL incidence, and that CLL incidence among the offspring of the Asian immigrants remained significantly lower compared to the local non-Hispanic whites. These observations together suggest that genetic factors are mainly responsible for this difference in CLL incidences, but the key molecular players remain to be identified.

MicroRNA (miRNA) are non-coding, single-stranded RNA (usually 20-23 nucleotides) with diverse biological functions. They can modulate the expression of genes at the post-transcriptional level and are involved in cancer, apoptosis, and cell metabolism.[14-16] In CLL cells, miRNA has been shown to interact with BCL2 (B-cell lymphoma-2) and TP53 (tumor protein p53) and might play a role in CLL pathogenesis.[17,18] In a study of 56 patients with CLL/SLL (small lymphocytic lymphoma), an overexpression of two miRNA, miR-21 and miR-155, was seen in most samples analyzed.[19] Loss of miR-15a and miR-16-a, associated with a deletion of chromosome 13q, could lead to leukemogenesis.[20,21] A study found that the expression of miR-15a, miR-16-1, miR-181a, miR-181b, and miR-29b in Chinese CLL patients was lower than in healthy donors.[22] Also, the expression of miR-29b and miR-181a/b was significantly correlated to the immunoglobulin heavy-chain variable region (IGHV) mutational status,[18,23] which is a strong prognostic indicator for CLL. Another study found that low expression of miR-223, miR-29c, miR-29b and miR-181, along with unmutated IGHV and high expression of ZAP-70 (Z chain-associated protein kinase 70) was associated with CLL progression.[18] Thus, it is evident that miRNA play an important role in CLL development, but it remains unclear if there are differentially expressed miRNA that could account for the difference in CLL incidence in Asia and western countries. Interestingly, analysis of miRNA expression in lymphoblastoid cell lines derived from Northern/Western European and from Nigeria Yoruba ethnic groups revealed a significant difference in miRNA expression between these two ethnic groups with an interesting correlation with drug sensitivity phenotypes.[24]

Based on the observations that miRNA could significantly affect CLL development and disease progression, and that population differences in miRNA expression have been detected in different ethnic groups, we hypothesized that the B lymphocytes from Asian and Western individuals might have differential expression of certain key miRNA which play an important role in B-cell survival and proliferation, thus contributing to the difference in CLL incidence in these two ethnic groups. This study was designed to test this possibility by using B lymphocytes isolated from Asian and Western individuals for miRNA analysis and functional study.

Methods

Primary B lymphocytes and cell lines

In the present study, CLL peripheral blood samples of Asian and Western CLL patients were obtained from Sun Yat-sen University Cancer Center (Guangzhou, China) and the University of Texas MD Anderson Cancer Center (Texas, USA), respectively. All patients were diagnosed according to the National Cancer Institute Criteria.[25] Informed consents under research protocols approved by the Institutional Review Board (IRB) of Sun Yat-sen University Cancer Center and MD Anderson Cancer Center were obtained from all patients before the collection of blood samples. CLL peripheral blood mononuclear cells (PBMC) samples with leukemia cells ≥70% were selected for this study. CLL cells were isolated from blood samples as previously described.[26] Normal PBMC were isolated from the buffy coats of blood samples from normal healthy individuals who donated blood at the regional blood banks. These samples were obtained in an anonymous fashion without personal information. After Ficoll-PaqueTM PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient centrifugation, normal $CD19^+$ B cells were purified using CD19 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Direct purification of $CD19^+$ B cells from PB was accomplished using EasySep™ Direct Human B-Cell Isolation Kit (Stem Cell Technologies, Cat #19674). The purity of B lymphocytes was analyzed by flow cytometry to confirm that more than 95% of the purified cells were $CD19^+/CD3^-$ (*Online Supplementary Figure S1A*). Normal blood samples were analyzed by sensitive flow cytometry to confirm their negativity for monoclonal B lymphocytosis (*Online Supplementary Figure S1B-D*). Two previously published CLL cell lines (MEC1 and MEC2)[27] were cultured in RPMI 1640 with glutamine (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum. HEK293T cells were cultured in DMEM (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum.

Microarray assays and data analysis

Total RNA was extracted from $CD19^+$ cells of CLL and healthy donors using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA was subjected to analysis of microRNA expression using the Affymetrix GeneChip® miRNA 3.0 Array. The detail procedures are provided in the *Online Supplementary Appendix*. The differences between samples were analyzed using the SAM (significance analysis of microarray) R package. The criteria for identifying differential expression between miRNA were a q value <0.05 and more than 2-fold change (FC >2.0 or <0.5). The microarray datasets of miRNA expression of this study have been deposited in the Gene Expression Omnibus database (GEO, GSE216258). Comparison analyses were performed using samples from four different groups including two CLL groups (Asian and Western) and two normal control groups (healthy donors from healthy Asian or Western individuals). Details of data comparisons and statistical analyses are described in the respective figure legends and in the *Online Supplementary Appendix*.

Vectors and oligonucleotides transfection

Lentiviral miArrest™ miRNA inhibitor vector for miRNA-4485 (Cat. # HmiR-AN2125-AM03, Genecopoeia, Guangzhou, China) and miExpress™ miRNA expression precursor for miR-4485 (Cat. # HmiR1168-MR03, Genecopoeia) were transduced into proper host cells. The transduced cells were selected with puromycin (Invivogen, San Diego, USA) or hygromycin (Invitrogen, Carlsbad, CA, USA) for 2-3 weeks to obtain cells with stable overexpression or low expression of miR-4485. MicrON miR-4485 mimic (dsRNA oligo, Cat. # miR10019019) and micrOFF miR-4485-3p inhibitor (single-stranded oligonucleotide, Cat. # miR2160621085914) were obtained from RiboBio Co., Ltd (Guangzhou, China). The miRNA mimic and inhibiting oligos were transfected into cells using a riboFect™ CP Transfection Kit as described in the *Online Supplementary Appendix*.

Detailed description of experimental procedures, data analysis, and statistical test is provided in the *Online Supplementary Appendix* and detailed information on antibodies used in the western blot is provided in *Online Supplementary Table S5*.

Results

Differential microRNA expression in primary chronic lymphocytic leukemia cells *versus* normal B-lymphocytes in Asian and Western individuals

The study design to reveal the potential differences in miRNA expression in primary CLL cells *versus* normal B-lymphocytes of Asian and Western individuals is illustrated in Figure 1A. In order to compare the microRNA expression profiles in the B lymphocytes and identify the miRNA that were differentially expressed in these two ethnic groups, total RNA was purified from B lymphocytes isolated from the blood samples of the following four groups of individuals: healthy Asians (n=9), healthy Western individuals (n=6), Asian CLL patients (n=22 including six patient samples in the initial GeneChip miRNA array analysis and 16 additional samples in the validation study), and Western CLL patients (n=9). The demographic and clinicopathological information of the CLL patients is summarized in the *Online Supplementary Table S1*. The global miRNA expression of each individual sample was profiled using the Affymetrix GeneChip® miRNA 3.0 Array. Using the t-distributed stochastic neighbor embedding (t-SNE) and unsupervised hierarchical clustering heatmap visualization, we showed that the four groups exhibited clearly distinguishable miRNA expression profiles (Figure 1B, C; *Online Supplementary Figure S2A-C*). Uniform manifold approximation and projection (UMAP) analysis also showed that each group had its own UMPA distribution pattern (*Online Supplementary Figure S2D*). Multivariate analyses including unsupervised principal component anlysis and supervised orthogonal partial least-squares discrimination analysis (OPLS-DA) revealed clear differences in miRNA expression in term of separation trend and clustering among the four groups (Figure 1D; *Online Supplementary Figure S3A, B, D, E, G, H, J, K*). We also used a permutation test with 200 random runs permutations to evaluate the reliability of our analyses. As shown in Figure 1D (right panel) and the *Online Supplementary Figure 3C, F, I, L*, the intercept of the Q2 regression line with the Y-axis was less than 0, indicating the OPLS-DA model was reliable and not overfitted.

Figure 1E shows a significant difference in miRNA expression in the primary leukemia cells from six Asian CLL patients and normal B lymphocytes from nine Asian healthy donors, using a FC>2 and a *q* value <0.05 as the criteria for statistical significance. The detail miRNA expression data and *q* values are shown in *Online Supplementary Table S2*. Similarly, the common miRNA expressed in the primary leukemia cells from nine Western CLL patients were also significantly different from the common miRNA expressed in the normal B lymphocytes from the healthy Western donors (Figure 1F; *Online Supplementary Table S3*). These data together showed that the differentially expressed miRNA between CLL and normal lymphocytes of the Asian samples were substantially different from that of the Western samples. Some of these differentially expressed miRNA such as miR-1295 and miR-4485 appeared in both ethnic groups (indicated by the red arrows in Figure 1E, F). These miRNA, either common in two ethnic groups or unique to a specific group, were further analyzed to identify candidate miRNA that might potentially contribute to the different CLL incidences between the two ethnic groups.

Identification of microRNA that potentially contribute to low chronic lymphocytic leukemia incidence in Asians

In order to identify the miRNA that might contribute to different CLL incidences in Asian and Western groups, we used Venn diagram and heatmap to visualize the differential miRNA expression profiles in normal lymphocytes and CLL cells of each ethnic group, using miRNA expression in normal B lymphocytes from healthy Western donors as the control for comparison against the other three groups. Because CLL incidence is high in the Western population, we reasoned that if a miRNA functioned to promote CLL development, it would be highly expressed in the CLL samples of both ethnic groups and would also be relatively lower in the normal lymphocytes of the Asian group compared to that of the Western group (low pro-CLL factor in Asians). Conversely, if a miRNA functioned as a CLL suppressor, its expression would be low in the CLL samples of both ethnic groups and also relatively low in the normal lymphocytes of the Western group compared to that of the Asian group. Using this analytical logic and a 2-FC (FC >2.0 or <0.5) in miRNA expression (increase or decrease) with a *q* value <0.05 as the cutoff criteria, we identified only a single microRNA (miR-4485) that met the criteria as a potential molecule that could promote CLL development but with relatively

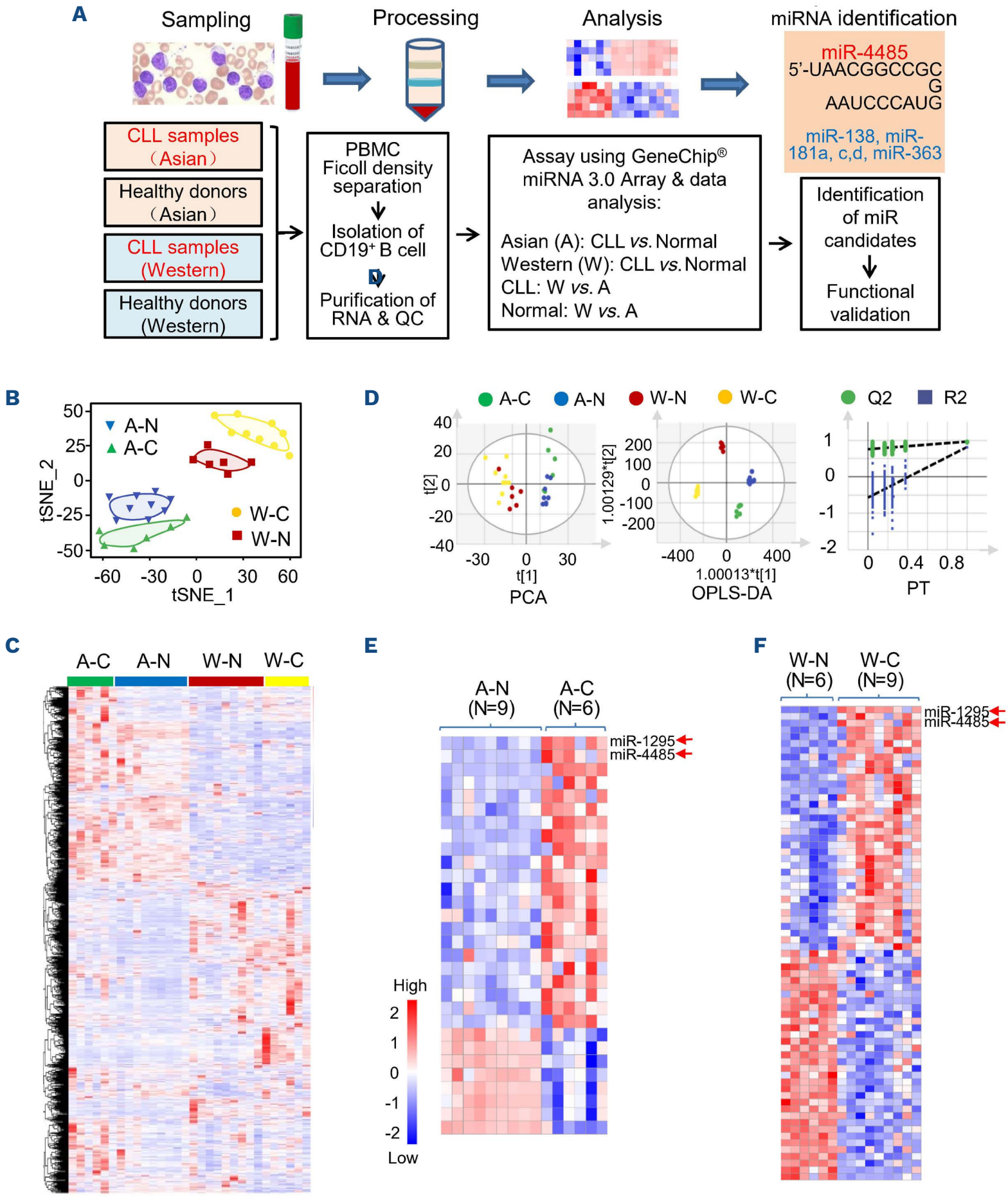


Figure 1. GeneChip® microRNA array analysis of microRNA expression profiles in primary chronic lymphocytic leukemia cells and normal B lymphocytes from Asian and Western individuals. (A) Schematic illustration of the study design, experimental approaches, and data analyses. (B) T-distribution stochastic neighbor embedding (t-SNE) plot showing sample clustering according to microRNA (miRNA) expression profiles. (C) Unsupervised hierarchical clustering of miRNA in different groups of samples. Each row indicates a miRNA, each column indicates a sample. The miRNA-clustering tree is on the left. (D) Score plots of the principal component analysis (PCA) model were set up using data from Asian CLL (A-C) (green plots) and Asian Normal (A-N) (blue plots), Western Normal (W-N) (red plots), Western CLL (W-C) (yellow plots), R^2X=0.585; the score plots of the orthogonal partial least-squared discrimination analysis (OPLS-DA) models discriminated the indicated four groups, and was confirmed by permutation test (PT). R^2X=0.854, R^2Y=0.976, Q^2=0.852. (E) Heatmap revealing the differentially expressed miRNA in chronic lymphocytic leukemia (CLL) cells *versus* normal B lymphocytes from Asian individuals, using 2-fold differential expression with a *q* value <0.05 as the cutoff values. (F) Heatmap showing the differentially expressed miRNA in CLL cells *versus* normal B lymphocytes from Western individuals. The miRNA with at least 2-fold differentially expressed levels with a *q* value <0.05 are shown.

Table 1. Quantitation of changes in microRNA expression in chronic lymphocytic leukemia cells and normal B lymphocytes from Asian and Western individuals.

MicroRNA name	FC (*q* value) Asian-CLL Asian-N	FC (*q* value) Western-CLL Western-N	FC (*q* value) Asian-N Western-N	FC (*q* value) Asian-CLL Western-CLL
miR-4485	9.49 (<0.001)	2.34 (0.008)	0.01 (<0.001)	0.05 (<0.001)
miR-181d	0.24 (0.007)	0.43 (0.030)	6.85 (<0.001)	3.86 (<0.001)
miR-181c	0.13 (0.007)	0.10 (<0.001)	2.98 (<0.001)	3.89 (0.005)
miR-363	0.22 (0.007)	0.16 (<0.001)	2.67 (<0.001)	3.66 (0.003)
miR-138	0.06 (<0.001)	0.06 (<0.001)	2.52 (0.006)	2.00 (0.040)
miR-181a	0.11 (0.001)	0.08 (<0.001)	2.07 (0.006)	1.48 (0.072)

Asian-CLL: microRNA expression in chronic lymphocytic leukemia (CLL) cells from Asian patients; Asian-N: microRNA expression in normal B lymphocytes from healthy Asians; Western-CLL: microRNA expression in CLL cells from Western patients; Western-N: microRNA expression in normal B lymphocytes from healthy Westeners. FC: fold change. The values in the table show the ratios of miR expression between the indicated ethnic samples, and the numbers in paratheses indicate the respective *q* values.

low expression in Asians (Figure 2A). Among the 84 miRNA with low expression in the normal B lymphocytes of Asian origin, only miR-4485 was highly expressed in CLL cells of both ethnic groups. Of note, similarly, although miR-1295 was upregulated in CLL cells of both ethnic groups as indicated in Figure 1E, F by the red arrows, its expression in Asian normal B lymphocytes was similar to that of the Western samples (Figure 2E, left panel; *Online Supplementary Tables S2* and *S3*), suggesting that miR-1295 was a potential pro-oncogenic molecule but could not account for the low CLL incidence in Asians.

The Venn diagram also revealed five miRNA (miR-181d, miR-181c, miR-363, miR-138 and miR-181a) that met the criteria as potential CLL suppressors with low expression in CLL cells of both ethnic groups and higher expression in the normal B lymphocytes from healthy Asian individuals compared with the normal B lymphocytes from healthy Western individuals (Figure 2B). The expression profiles of these six miRNA in each group were further analyzed using volcano plots and visualized by heatmaps (Figure 2C-E). The results revealed that miR-4485 was upregulated and miR-181d, miR-181c, miR-363, miR-138, miR-181a were downregulated in all CLL samples of both ethnic groups (Figure 2C, D). Importantly, miR-4485 was significantly downregulated, while miR-181d, miR-181c, miR-363, miR-138, and miR-181a were highly elevated in the normal B lymphocytes from Asian healthy individuals (Figure 2E). The quantitative data for these comparisons and the respective *q* values are summarized in Table 1, which shows the ratios of miR expression in CLL cells/normal B lymphocytes in the two ethnic groups and the ratios of miR expression in Asian/Western normal B lymphocytes and CLL patients. Of special note, the expression of miR-4485 was 9.5-fold and 2.3-fold higher in Asian and Western CLL patients compared to the respective normal B lymphocytes of the same ethnic groups, whereas its expression in the normal lymphocytes from Asian healthy individuals was 83-fold lower than that in the normal lymphocytes from healthy Western individuals.

High expression of miR-4485 promotes chronic lymphocytic leukemia cell proliferation

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was first used to confirm the low expression of miR-4485 in Asian normal B lymphocytes and its upregulation in CLL cells, using samples from nine healthy Asian individuals and 12 Asian CLL patients. As shown in Figure 3A, miR-4485 expression was low in primary normal B lymphocytes and significantly increased in the CLL samples ($P<0.05$; Figure 3A, left panel). The expressions of miR-138, miR-181c, miR-363, miR-181a and miR-181d which was found to be upregulated in normal lymphocytes in miRNA array analysis was also validated using qRT-PCR analysis. The results also showed that the levels of miR-138, miR-181c, miR-363, miR-181a and miR-181d were significantly higher in the Asian normal B lymphocytes than in the primary CLL cells from Asian patients (Figure 3A, right 5 panels), similar to that observed in miRNA array analysis and consistent with their presumed tumor suppressor function. Consistently, analysis of two publicly available datasets GSE108901 and GSE66186, which contained miRNA expression data mainly from Western CLL patients and Western normal individuals, also showed higher expression of pro-CLL miR-4485 and lower expression of the CLL-suppressive miRNA in CLL patients compared with healthy individuals (*Online Supplementary Figure S4*).

Since the five miRNA identified in Figure 2B (miR-181d, miR-181c, miR-363, miR-138, and miR-181a) with putative anti-CLL function had been reported previously,[23,28-34] whereas miR-4485 was the only microRNA identified as a putative CLL-promoting molecule with high expression in CLL cells in both ethnic groups and a downregulation in Asian normal B lymphocytes, we thus focused our efforts on investigating the role of miR-4485. In order to investigate the functional impact of miR-4485 on CLL cells, we first used a lentiviral

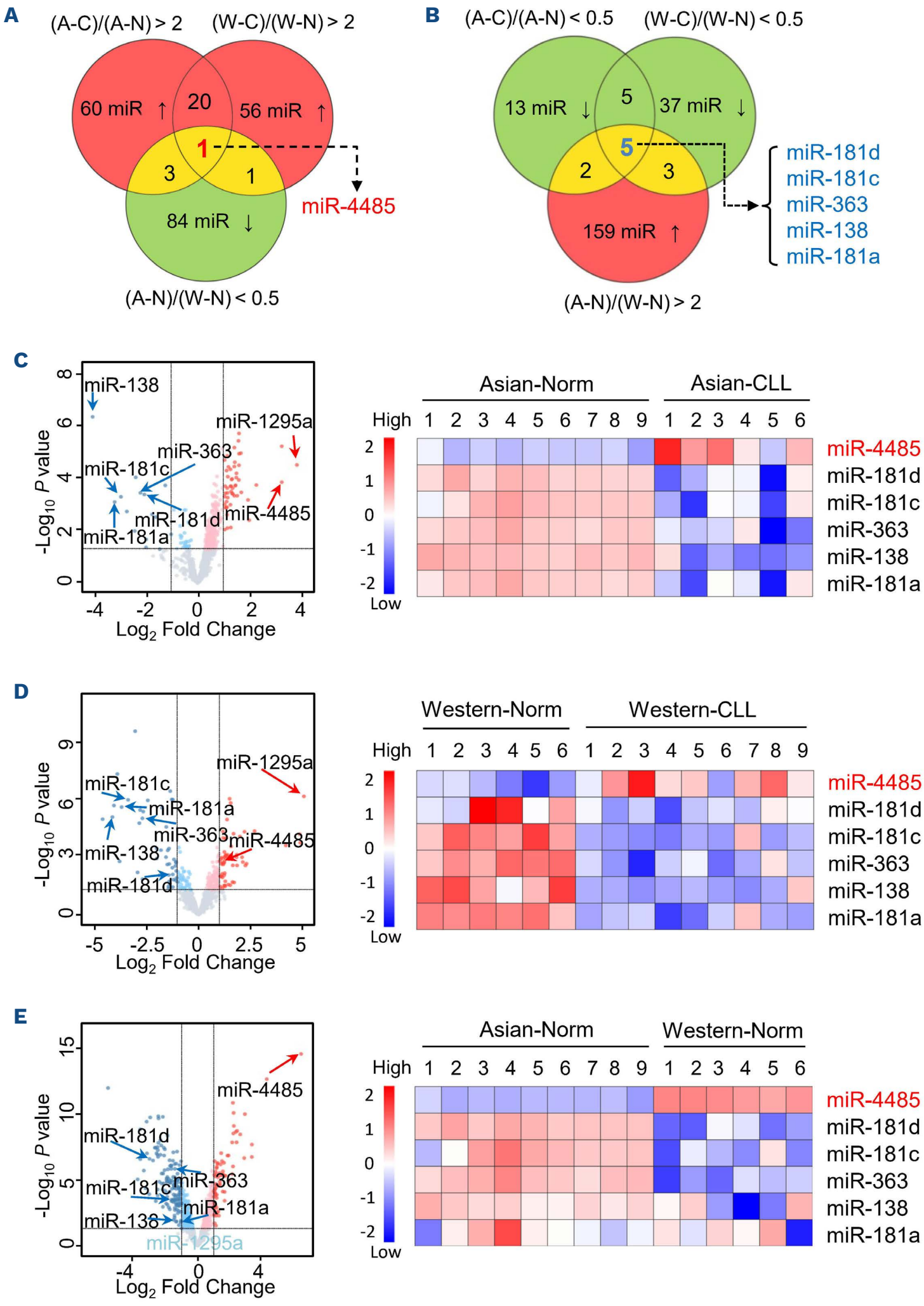


Figure 2. Identification of microRNA differentially expressed in chronic lymphocytic leukemia cells and normal B lymphocytes from Asian and Western individuals. (A) Venn diagram revealing the differentially expressed microRNA (miRNA) that exhibited at least 2-fold difference (ratio >2 or <0.5) in miRNA expression with a *q* value <0.05 for the indicated comparison groups: Asian chronic lymphocytic leukemia (CLL) (A-C) *versus* Asian Normal (Norm) (A-N); Western-CLL (W-C) *versus* Western-Norm (W-N); Asian-Norm (A-N) *versus* Western-Norm (W-N). The red color represents the elevated miRNA expression with a ratio >2.0 for the indicated groups, while the green color shows lower expression with a ratio <0.5 for the indicated groups. The red number "1" in the center of the

Continued on following page.

diagram indicates the only miRNA (miR-4485) that was highly expressed in all CLL samples of both Asian and Western groups but was significantly lower in Asian normal B lymphocytes compared with Western normal B lymphocytes. (B) Venn diagram showing the differentially expressed miRNA that exhibited at least 2-fold difference (ratio >2.0 or <0.5) in miRNA expression with a *q* value <0.05 for the comparison groups. The color codes are the same as in (A). The blue number "5" in the center of the diagram indicates 5 miRNA that were downregulated in CLL cells of both Asian and Western groups but highly expressed in Asian normal B lymphocytes compared with Western normal B lymphocytes. (C-E) Left panels: volcano plots showing the differentially expressed miRNA in Asian-CLL compared to Asian-Norm (C), Western-CLL compared to Western-Norm (D), and Western-Norm compared to Asian-Norm (E). Differentially expressed miRNA were identified that meet fold change (FC) >2 (log2 FC>1) and *P*<0.05 (-log10 *P*>1.30) calculated using two-tailed Student's *t* test. Significant upregulated and downregulated miRNA are respectively indicated with red and blue points that exhibit at least 2-fold change (x axis; log2 FC>1) and statistical significance (y axis; -log10 *P*>1.30). The top selected miRNA are marked with annotation on the plot; right panels: heatmaps illustrating the expression of the single microRNA (miR-4485) identified in (A) and the five miRNA (miR-181d, miR-181c, miR-363, miR-138, and miR-181a) identified in (B) in Asian CLL cells and Asian normal lymphocytes (C), in Western CLL cells and Western normal lymphocytes (D), and in Asian and Western normal B lymphocytes (E). N: normal B lymphocytes; C: CLL; A: Asian; W: Western.

vector (pEZX-AM03) expressing miR-4485 antagonist to transfect two CLL cell lines, MEC1 and MEC2 (both with a high level of miR-4485), and then tested the effect of miR-4485 inhibition on cell proliferation at 24 hour (hrs), 48 hrs, and 72 hrs. There was a significant decrease in cell proliferation after MEC1 or MEC2 cells were transfected to express miR-4485 antagonist, whereas transfection using the control vector did not affect cell proliferat6ion (Figure 3B). The effect of miR-4485 on CLL cell proliferation was further tested using miR-4485 mimic or inhibiting oligos in MEC1 and MEC2 cells. As shown in Figure 3C, cell proliferation was significantly enhanced by miR-4485 mimic compared to the oligo control, whereas the miR-4485 inhibitor significantly reduced cell proliferation in both cell lines. We also used HEK293T cell line with a low level of endogenous miR-4485 for infection with a lentivirus containing miR-4485 overexpression vector (pEZX-MR03) or with the control vector. The increased expression of miR-4485 in HEK293T cells led to a moderate but significant increase in cell proliferation (*P*<0.01; Figure 3D, E). Consistently, miR-4485 mimic also significantly increased HEK293T cell proliferation, whereas miR-4485 inhibitor markedly reduced cell growth (Figure 3F).

Suppression of TGR5 and activation of ERK1/2 pathway by miR-4485

In order to investigate the potential mechanisms by which miR-4485 promoted cell proliferation that might contribute to CLL development, we first performed target prediction analysis using multiple web tools (TargetScan, miRwalk, miRPathDB, miRDB) to generate a list of potential target genes by each web tool, and used Venn diagram to reveal the common candidate genes shared by all four sets of analyses (Figure 4A). We found six genes (*ASB7, CTDSPL2, FBXO32, JOSD1, MED1, TGR5*) that were likely regulated by miR-4485. qRT-PCR was then used to measure the expression of these six genes in $CD19^+$ normal B lymphocytes purified from healthy Asian donors (n=7) in comparison with primary CLL cell isolated from Asian patients (n=11). We found that only *TGR5* gene expression was significantly suppressed in CLL cells compared with normal lymphocytes (Figure 4B), whereas the expression levels of the other five genes were similar in normal lymphocytes and leukemia cells (*Online Supplementary Figure S5A-E*). We also compared the expression of these six genes using a dataset from Gene Expression Omnibus (GEO, GSE66117), which contained RNA expression data from 45 CLL patients and five normal individuals. The results consistently showed that TGR5 expression was lower in CLL cells than in normal lymphocytes, whereas the expression of the other five genes was similar in CLL and normal lymphocytes (Figure 4C; *Online Supplementary Figure S5F-J; Online Supplementary Table S4*). Based on these observations, we focused our study on the potential regulation of TGR5 by miR-4485, using a luciferase reporter assay in which HEK293T cells were co-transfected with a miR-4485 mimic and a Dual-Glo® luciferase assay system containing either the wild-type 3'UTR (untranslated region) of TGR5 with a miR-4485 binding sequence or with a mutated miR-4485 binding sequence (Figure 4D). The results showed that miR-4485 expression significantly suppressed the luciferase activity in cells containing the wild-type miR-4485 binding sequence, whereas such inhibition was not observed in cells transfected with the mutated miR-4485 binding sequence (Figure 4D).

In order to further test the ability of miR-4485 to down-regulate TGR5 expression, we transfected HEK293T cells with a lentiviral-mediated miR-4485 overexpression vector, and measured TGR5 mRNA and protein expression by qRT-PCR and western blot analyses, respectively. The results showed that overexpression of miR-4485 caused a significant decrease in TGR5 mRNA and protein (Figure 5A, B). Conversely, transfection of HEK293T cells with a lentiviral vector expressing a miR-4485 antagonist (miR-4485 inhibitory sequence) led to a significant increase in TGR5 mRNA and protein expression (Figure 5C). In order to further test if miR-4485 could regulate TGR5 expression in CLL cells, MEC1 and MEC2 cells were transfected with miR-4485 mimic, miR-4485 inhibitor, or their respective control oligos, and their effect on TGR5 expression was measured. The results showed that miR-4485 mimic caused a decrease in TGR5 protein, whereas miR-4485 inhibitor enhanced TGR5 protein expression (Figure 5D; *Online Supplementary Figure*

S6A). The upregulation of *TGR5* mRNA and protein was also consistently observed in primary CLL cells transfected with miR-4485 inhibitor (Figure 5E).

Since the roles of miR-4485 and its target gene TGR5 in CLL development largely remained unknown, we then examined the potential effect of miR-4485 on the expression of molecules such as STAT3, Bcl-2, Bcl-xL, MEK1/2 , ERK1/2 and c-Jun which are important for CLL cell survival and proliferation. Among all the molecules tested, overexpression of miR-4485 in HEK293T cells caused a decrease in TGR5 pro-

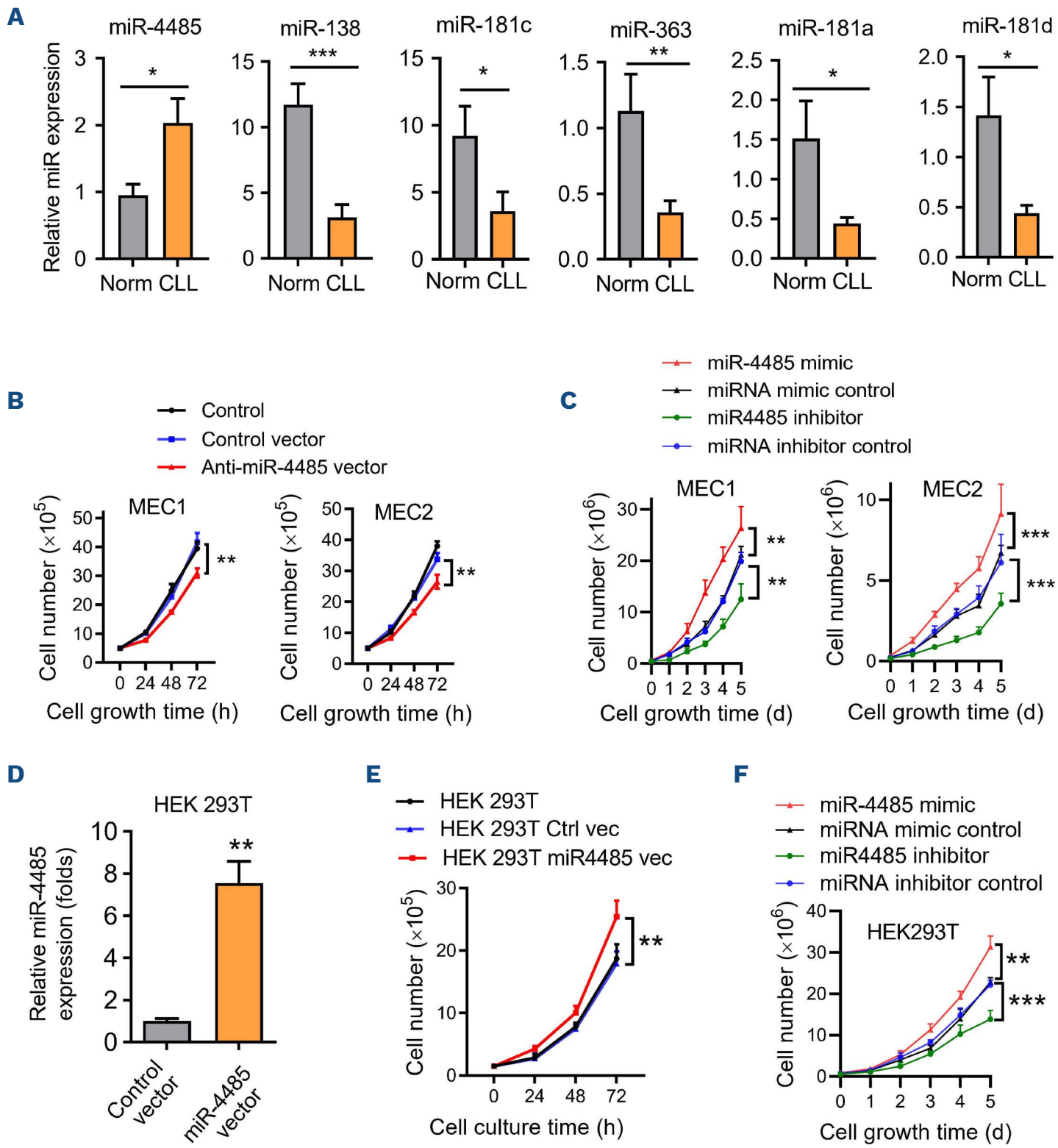
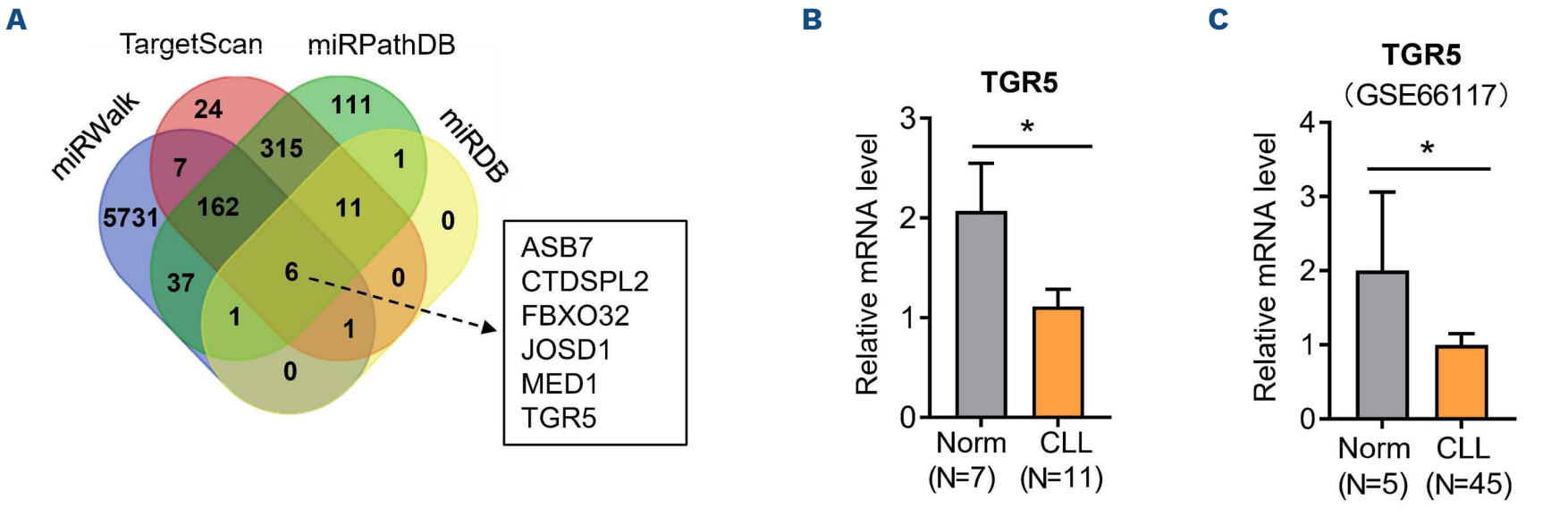


Figure 3. Quantitative analysis of key microRNA expression and the impact of miR-4485 on cell proliferation. (A) Expression of miR-4485, miR-138, miR-181c, miR-181d, miR-363, miR-181a in primary chronic lymphocytic leukemia (CLL) cells from patients and normal B lymphocytes from Asian individuals. The levels of microRNA (miRNA) were quantified by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and presented as mean ± standard deviation (SD) (Norm, N=9; CLL, N=12 for analysis of miR-4485, miR-138 and miR-181c; Norm, N=6; CLL, N=10 for miR-181d, miR-363 and miR-181a). (B) Effect of miR-4485 inhibition on CLL cell growth. MEC1 cells and MEC2 cells were transfected with a miArrest™ miRNA inhibitor vector (pEZX-AM03-based) containing miR-4485 antagonist sequence or the control vector as indicated, and cell numbers were counted at 24 hours (hrs), 48 hrs, and 72 hrs. (C) MEC1 and MEC2 cells were transiently transfected with miR-4485 mimic or inhibitor oligos and their corresponding negative control oligos; 48 hrs after transfection, cell numbers were counted every 24 hrs. (D, E) HEK293T cells were infected with lentiviral containing miR-4485 expression vector (pEZX-MR03-based) or the control vector, and the level of miR-4485 expression was measured by qRT-PCR (D) and cell numbers were quantified after 24 hrs, 48 hrs, and 72 hrs (E). (F) HEK293T cells were transiently transfected with miR-4485 mimic or inhibitor oligonucleotides and their corresponding negative controls, 48 hrs after transfection, cell numbers were counted every 24 hrs. Two-way ANOVA analysis was used to determine the significance of the proliferation differences between 2 groups. The mean value ± SD of 3 experiments is shown. ***P*<0.01; ****P*<0.001.

tein expression and an increase in ERK1/2 phosphorylation without an increase in total ERK1/2 protein. The expression of phosphorylated STAT3, total STAT3 protein, Bcl-2, and Bcl-xL remained unchanged (Figure 5B). Conversely, inhibition of miR-4485 consistently led to an upregulation of TGR5 mRNA and protein (Figure 5C), a decrease of phosphorylated ERK1/2 and downstream c-Jun and p-c-Jun proteins in MEC-1 and MEC-2 cells, while transfection of miR-4485 mimic inhibited TGR5 expression and activated ERK1/2 and its downstream p-c-Jun and c-Jun (Figure 5D; *Online Supplementary Figure S6A*). Inhibition of miR-4485 in primary CLL cells also decreased phosphorylation of ERK1/2 and c-Jun (Figure 5E). In order to evaluate the relationship between TGR5 expression and ERK1/2 activation, we first tested the effect of ERK1/2 specific inhibitor SCH772984 on TGR5 expression in MEC1 and MEC2 cell lines and in primary CLL cells, and found that inhibition of ERK1/2 led to an increased expression of TGR5 (Figure 6A, *Online Supplementary Figure S6B*), suggesting a negative regulation of TGR5 by ERK1/2. Importantly, TGR5 overexpression in MEC1 and MEC2 cells or activation of TGR5 with a TGR5 agonist treatment in primary CLL cells caused a decrease in ERK1/2 phosphorylation (Figure 6B, *Online Supplementary Figure S6C, D*), suggesting an inhibitory effect of TGR5 on ERK1/2 activation. This effect was



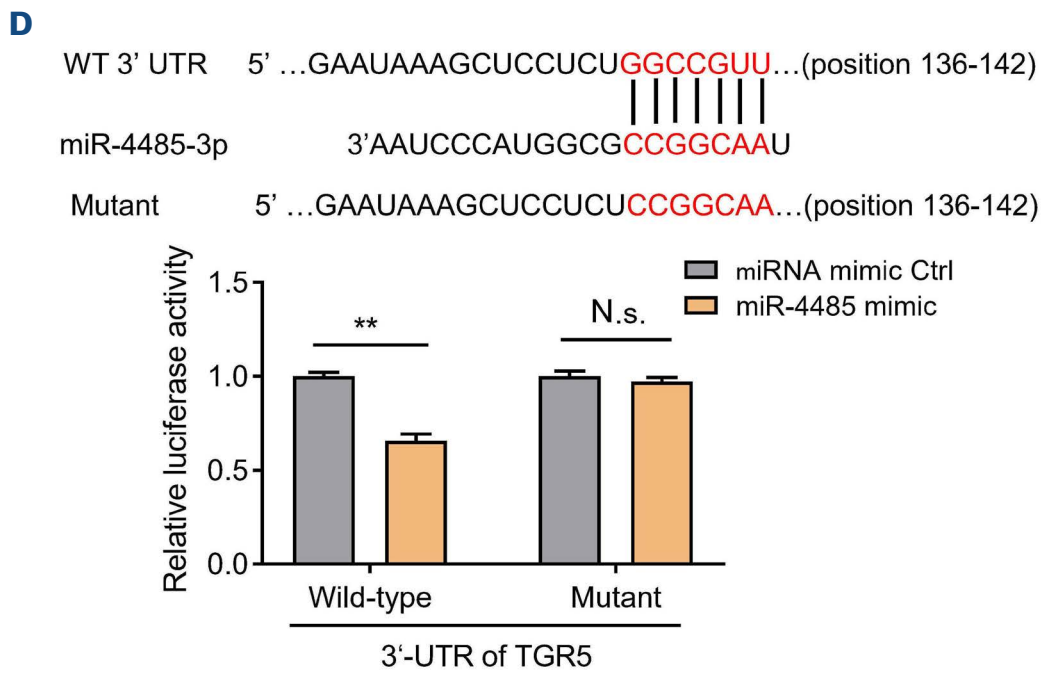


Figure 4. Identification of TGR5 as the target molecule of miR-4485. (A) Venn diagram revealing the putative target molecules of miR-4485 predicted by mirWalk, TargetScan, miRPathDB, and miRDB. Six genes (*TGR5, ASB7, CTDSPL2, FBXO32, JOSD1, MED1*) were commonly identified by all 4 webtools as potential targets for miR-4485. (B) Expression level of *TGR5* mRNA, quantified by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), in primary chronic lymphocytic leukemia (CLL) cells from Asian patients (N=11) and normal B lymphocytes from Asian healthy donors (N=7, mean ± standard deviation [SD]); **P*<0.05. (C) Comparison of *TGR5* gene expression in primary leukemia cells from CLL patient and normal B lymphocytes from healthy donors, using the GSE66117 dataset; data are presented as mean ± SD; **P*<0.05. (D) Key nucleotide sequences of the luciferase reporters: the wild-type sequence of *TGR5* 3' untranslated region (3'UTR) containing potential miR-4485 binding site is shown on the upper line; the complementary sequence of miR-4485 is shown in red, with corresponding miR-4485 sequence shown in the middle line; the mutated 3'UTR sequence containing 7 altered nucleotides (red color) are shown in the third line. The bar graph in the lower panel shows the results of luciferase assay using HEK293 cells co-transfected with miR-4485 mimic and the luciferase reporter containing either wild-type TGR5 3'UTR or the mutated TGR5 3'UTR; ***P*<0.01.

unlikely mediated by the canonical MEK/ERK1/2 pathway, since TGR5 did not cause any significant change in MEK or p-MEK (*Online Supplementary Figure S6C, D*). At the cellular level, TGR5 overexpression in MEC1 and MEC2 cells led to a significant decrease in cell proliferation (*Online Supplementary Figure S6E, F*), consistent with its inhibitory effect on ERK1/2 activation.

In order to further evaluate the potential role of TGR5 in mediating the regulation of miR-4485 on ERK1/2 and cell proliferation, we first used MEC2 cell line to generate stable TGR5-knockdown cells that exhibited an increase in ERK1/2 phosphorylation and cell proliferation (*Online Supplementary Figure S7A, B*), and then transfected the cells with miR-4485 inhibitory oligos to test its impact on ERK1/2 and cell proliferation. In MEC2 cells transfected with control small hairpin RNA (shRNA), inhibition of miR-4485 by inhibitory oligos consistently led to upregulation of TGR5 expression, suppression of ERK1/2 phosphorylation (Figure 6C), and a significant decrease in cell proliferation (*Online Supplementary Figure S7C*). In contrast, miR-4485 inhib-

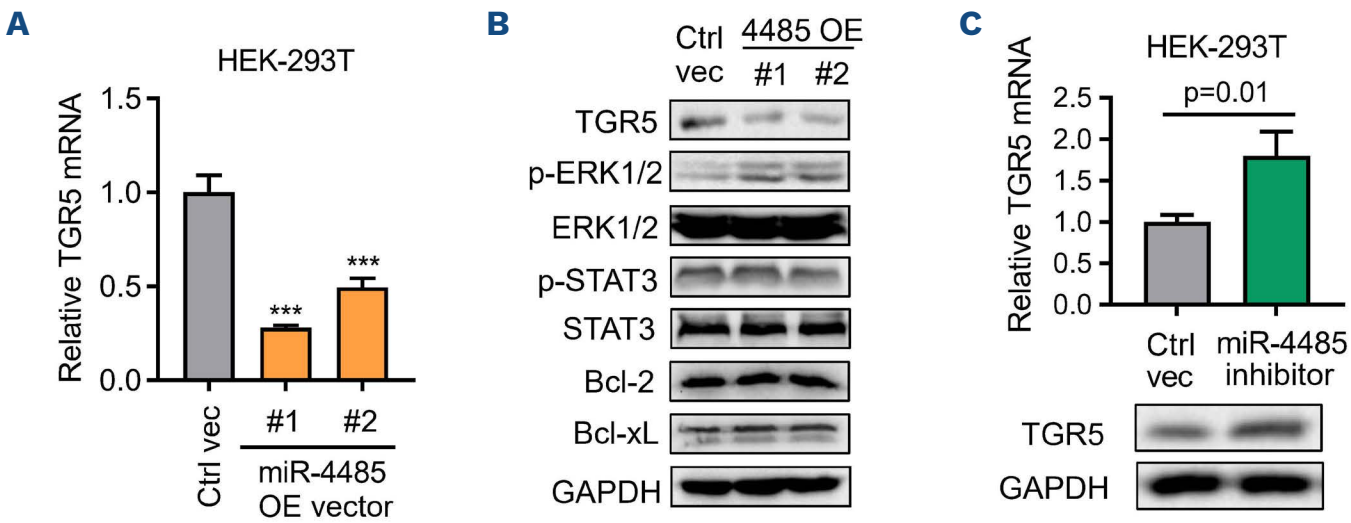


Figure 5. miR-4485 suppresses TGR5 expression and promotes phosphorylation of ERK1/2. (A) HEK293T cells were transfected with miR-4485 overexpression vectors, and 2 stable clones were selected for analysis of TGR5 expression by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (N=3, mean ± standard deviation [SD]). (B) HEK293T cells were transfected with miR-4485 overexpression vectors, and 2 stable clones were selected for analysis of protein expression by western blotting, using the specific antibodies for the molecules of interest as indicated. (C) HEK293T cells were transfected with a miArrest™ microRNA (miRNA) inhibitor vector (pEZX-AM03) containing an miR-4485 antagonist sequence. The expression of TGR5 mRNA and protein were measured by qRT-PCR and western blotting, respectively (N=3, mean ± SD). (D) MEC1 cells were transiently transfected with miR-4485 mimic or inhibitor, and their corresponding negative controls, and the expression levels of TGR5, phosphorylated MEK1/2 (Ser217/221), MEK1/2, phosphorylated ERK1/2 (Thr202/Tyr204), ERK1/2, phosphorylated-c-Jun (S63), and c-Jun was measured by western blot analysis. (E) Primary chronic lymphocytic leukemia (CLL) cells were transiently transfected with miR-4485 inhibitor or its corresponding negative control, the expression of TGR5 mRNA and protein were measured by qRT-PCR and western blotting, respectively. The expression of ERK1/2, phosphorylated ERK1/2, phosphorylated-c-Jun and c-Jun was also measured by western blot analysis. miRi Ctrl: miRNA inhibitor control; miR-4485i: miR-4485 inhibitor; miRm Ctrl: miRNA mimic control; miR-4485m: miR-4485 mimic. ***P<0.001.

itor could no longer induce TGR5 expression and did not suppress ERK1/2 phosphorylation in MEC2 cells with TGR5 knockdown by shRNA (Figure 6C), and its inhibitory effect on cell proliferation was also largely attenuated in MEC2 cell with TGR5 knockdown (*Online Supplementary Figure S7D, E*). These data together suggest that TGR5 might play an important role in mediating the effect of miR-4485 on ERK1/2 activation and cell growth, as illustrated in Figure 6D.

Discussion

It has been known for decades that the incidence of CLL is significantly lower in Asians compared to that in the Western population.[4-10] The fact that CLL disease frequency remains low in Asians despite living in the western countries for multiple generations suggests the existence of strong genetic factors that affect CLL development. However, the exact identities of such genetic factors remain largely unknown, although certain genes with general pro-CLL functions have been identified. For example, unmutated IGHV, p53 mutation, Bcl-2 overexpression, 17p deletion [Del (17p)], 11q deletion [Del (11q)], Trisomy 12, 13q deletion [Del (13q)], and low expression of miR-15/16 have been implicated to play certain roles in CLL development,[35-38] but these genetic events did not show differential occurrence between Asian and Western populations and thus could not account for the different CLL incidences in the two ethnic groups. Thus, further investigations are required to reveal the possible genetic differences between Asian and Western populations that could explain the population difference in CLL leukemogenesis, which would not only gain further insights into CLL biology, but would also provide a basis for developing new strategies for effective CLL treatment. Based on the significant role of miRNA in affecting CLL development,[39,40] the objectives of the current study were to identify candidate miRNA that might contribute to the different incidences of CLL in Asian and Western populations, and to investigate the relevant molecular mechanisms. Using the GeneChip® miRNA array analysis, we compared

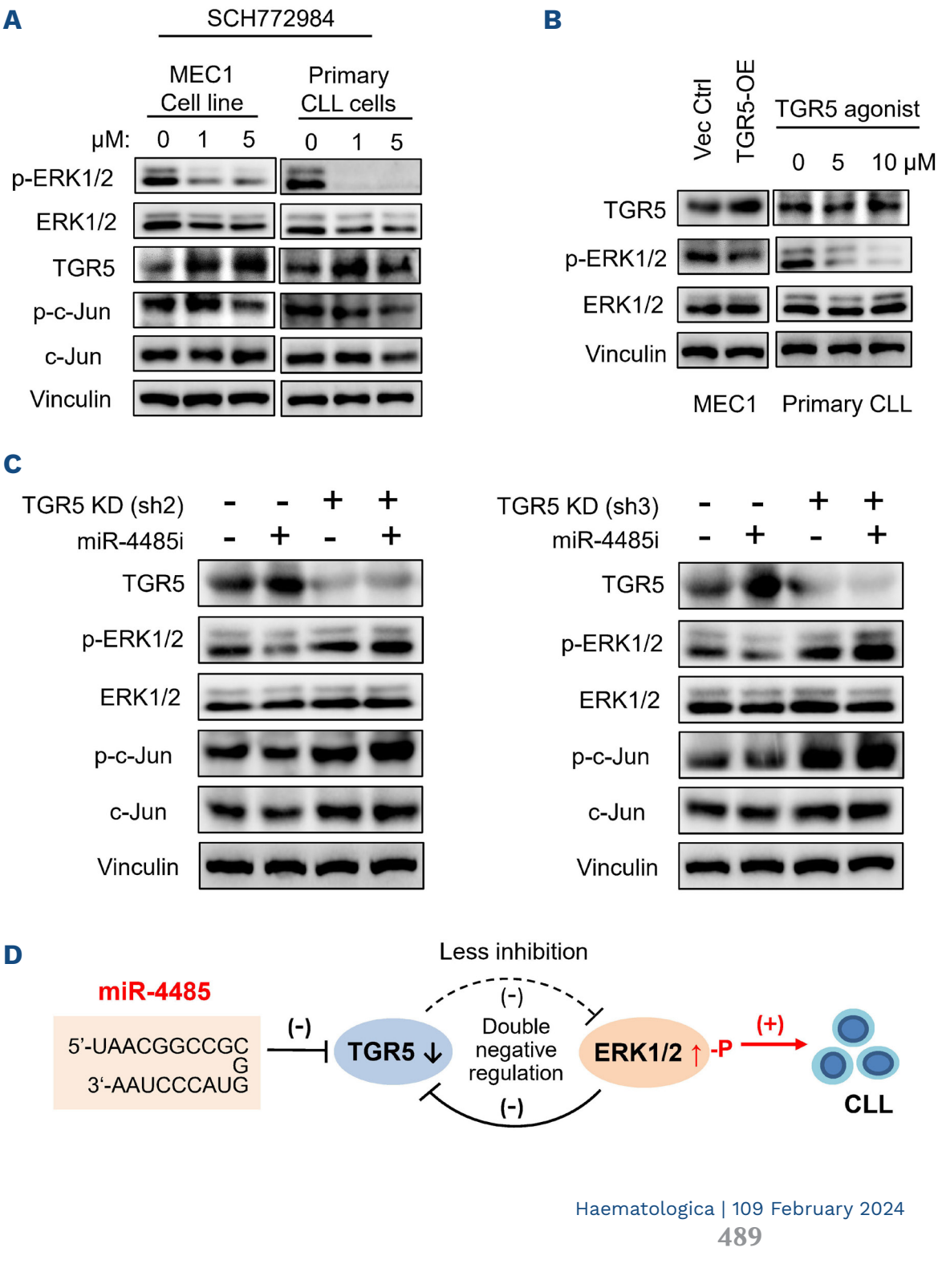


Figure 6. TGR5 plays a key role in mediating the effect of miR-4485 on ERK1/2 activation. (A) MEC1 cells and primary chronic lymphocytic leukemia (CLL) cells isolated from CLL patients were treated with or without SCH772984 (an ERK inhibitor) at 1-5 μM for 48 hours (hrs), and the indicated molecules were determined by western blot. (B) MEC1 cells were transduced with empty control vector or TGR5 expression vectors (TGR5-OE), and primary CLL cells were incubated with TGR5 agonist at indicated concentrations for 48 hrs. The cells were then collected for western blot analysis of indicated proteins. (C) MEC2 cells stably transduced with empty vector or TRG5 knockdown vectors (shTGR5-#2, shTGR5-#3) were transiently transfected with miR-4485 inhibitor (miR-4485i) or its negative inhibitor control, and cells were collected for western blot analysis of indicated proteins. (D) Schematic model illustrating possible mechanisms by which miR-4485 promotes CLL development through inhibiting TGR5 expression and thus decreasing its suppressive function, leading to an increase of ERK1/2 phosphorylation and enhancing CLL cell proliferation. The activation of ERK1/2 could also suppress TGR5 expression and thus further reduce its inhibitory effect on ERK1/2, leading to further activation ERK1/2. As such, TGR5 and ERK1/2 form a double-negative loop, which could amplify the pro-CLL effect of miR-4485. miR-4485i: miR-4485 inhibitor.

the global miRNA expression profiles in primary CLL cells and normal lymphocytes of the two ethnic groups, aiming to identify (i) pro-CLL miRNA with high expression in all CLL cells and lowest expression in Asian normal B lymphocytes (lower than Western normal B lymphocytes), and (ii) anti-CLL miRNA with low expression in all CLL cells and highest expression in Asian normal B lymphocytes (higher than Western normal B lymphocytes). Using this logical approach, we identified a single miRNA (miR-4485) in the first category and five miRNA (miR-181d, miR-181c, miR-363, miR-138, miR-181a) in the second category. It is likely that the low expression of pro-CLL miR-4485 and the high expression of the five anti-CLL miRNA in Asian normal B lymphocytes collaboratively or collectively contribute to the low CLL incidence in Asian population. Of note, although the tumor suppressive functions of miR-181d, miR-181c, miR-363, miR-138, and miR-181a have been reported previously,[23,28-34] the novelty of our study was the finding that these anti-CLL miRNA were expressed significantly higher in Asian normal B lymphocytes than in Western normal B lymphocytes, and this could decrease the chance of CLL development in the Asian population. In contrast, the expression of miR-4485 with pro-CLL function was higher in Western normal B lymphocytes than in the Asian normal B lymphocytes, and might likely contribute to the higher CLL incidence in the Western population.

The observations that miR-4485 expression was significantly higher in primary CLL cells of both Asian and Western patients compared to that in the normal B lymphocytes from healthy individuals of the respective ethnic groups suggest that this miRNA might potentially have a role in promoting CLL development. Indeed, we showed that inhibition of miR-4485 in two CLL cell lines resulted in a significant retardation of cell proliferation, whereas ectopic expression of miR-4485 in HEK293T cells led to an increase in cell proliferation, indicating the cancer promoting function of this miRNA. Importantly, we found that while the expression of miR-4485 was generally low in normal B lymphocytes, its level was particularly lower in Asian normal B lymphocytes compared to Western normal lymphocytes. Together, these novel findings suggest that the differential expression of miR-4485 in the normal B lymphocytes of Asian *versus* Western groups likely contributed to the different CLL incidences in these two populations, although the exact mechanisms for its pro-CLL function remain to be further explored.

In human cells, miR-4485 is located on chromosome 11p15.4, and its function has not been well-characterized. Jima *et al*. used deep sequencing to analyze miRNA expression in normal B lymphocytes from the tonsils of healthy individuals in comparison with that in malignant B cells from lymphoma patients, and found higher expression of miR-4485 in malignant B cells.[41] A study by Sripada *et al*. showed that miR-4485 was translocated to mitochondria where it regulated the processing of mitochondrial 16S rRNA and affected mitochondrial functions and cellular metabolism in breast cancer cells.[42] Interestingly, the same study suggested that miR-4485 might function as a tumor suppressor in breast carcinoma since its expression was low in breast cancer tissues and ectopic expression of miR-4485 in breast cancer cells led to a decrease in tumor growth.[42] Thus, miR-4485 appeared to have tumor promoting or suppressor functions depending on cell type. In our study, we found that miR-4485 was highly expressed in primary leukemia cells from CLL patients and could promote cell proliferation in two CLL cell lines, suggesting that it might have a pro-oncogenic function in B lymphocytes, consistent with the observation in B-cell lymphoma.[41]

The exact mechanism by which miR-4485 promotes CLL would provide an important insight into the regulation of CLL development. Our study showed that TGR5, also known as G-protein coupled bile acid receptor 1 (GPBAR1), seems to be a direct target molecule of miR-4485. This conclusion is supported by multiple evidence: (i) luciferase assay showed that miR-4485 could suppress the luciferase reporter activity in a vector containing 3'-UTR of TGR5, (ii) ectopic expression of miR-4485 downregulated the expression of TGR5, (iii) transfection of CLL cells with miR-4485 inhibitor led to the upregulation of TGR5 expression in two CLL cell lines, and (iv) inhibition of miR-4485 in primary CLL cells also resulted in an elevated expression of TGR5. Several studies have shown that TGR5 is highly expressed in normal tissues, has the ability to regulate glucose and lipid metabolism and maintain energy homeostasis, inhibits inflammatory reactions, and might have a tumor-suppressing function.[43-45] Activation of TGR5 has been shown to suppress cell proliferation and migration through dephosphorylation of STAT3 in gastric cancer.[46] The tumor-suppressive effect of TGR5 was also observed in hepatocellular carcinoma, again through inhibition of STAT3 signaling pathway.[47] In our study, although we consistently found that miR-4485 could significantly downregulate TGR5 expression in CLL cells and HEK cells, the decrease in TGR5 did not cause any detectable changes in STAT3 protein level or its phosphorylation. Instead, we observed a significant increase in phosphorylation of the ERK1/2, suggesting that activation of the ERK1/2 signaling pathway might be a potential mechanism by which miR-4485 promote CLL development. TGR5 seems to play a key role in mediating the effect of miR-4485 on activation of ERK1/2 and promotion of cell proliferation, as a knockdown of TGR5 abolished such effect of miR-4485. As illustrated in Figure 6D, miR-4485 seems to exert its pro-CLL function through a double-negative mechanism with a double-negative amplification loop in the following fashion. miR-4485 negatively regulates the expression of TGR5 and thus reduces its negative impact on ERK1/2 phosphorylation, leading to activation of ERK1/2 and cell proliferation. This double negative effect could be further amplified by a double-negative regulatory loop between ERK1/2 and TGR5. Since inhibition of ERK1/2 could enhance TGR5 expression (Figure 6A), the activation of ERK1/2 would then suppress TGR5

expression and thus reduce its inhibitory effect on ERK1/2, leading to further activation ERK1/2. This double-negative loop could potentially provide a novel mechanism to amplify the pro-CLL effect of miR-4485.

Our study suggests that differential expression of miRNA in Asin and Western individuals may contribute to the different CLL incidents in the two ethnic populations, and provide novel insights into the regulatory mechanism of the miR-4485/TGR5/ERK1/2 axis in the context of CLL development. However, it should be noted that our study has several limitations including small sample size, a lack of longitudinal samples at various stages of CLL development, and potential batch effects on miRNA assays due to samples acquisition and analysis in two different geological locations, although every effort was made to keep the experimental conditions identical and to minimize the risk of batch effect. Because of limited number of samples, we have not performed analysis of several same samples in both countries to evaluate the magnitude of potential batch effect. Thus, the differential expression of miR-4485 and several other miRNA should be further validated in larger sample sizes in future study. A longitudinal follow-up with analysis of blood samples at different stages during CLL development has the advantage of identifying the critical time when abnormal expression of key miRNA and activation of the pro-CLL pathway may occur. This may potentially identify the miRNA markers related to disease initiation and progression and the potential underlying mechanisms and therapeutic targets. Also, it would be interesting to include samples from Western CLL patients who live in Asia and samples from Asian CLL patients who live in the Western country as crucial controls for ideal comparison. Thus, it would be important to conduct a prospective study with larger sample sizes and all necessary control groups, and with sufficient follow-up time to determine whether high miR-4485 is associated with increased risk of developing CLL.

In summary, through comparative analyses of the global miRNA expression profiles in primary CLL cells and normal B lymphocytes from Asian and Western individuals, we have identified miR-4485 with CLL-promoting function and miR-138, miR-181a, miR-181c, miR-181d, and miR-363 with tumor suppressor functions that were differentially expressed in Asian and Western individuals and likely contributed to the different CLL incidences in the two ethnic groups. The pro-CLL activity of miR-4485 is likely mediated by inhibition of TGR5 expression and thus reducing its tumor-suppressive effect on ERK1/2 phosphorylation, leading to ERK1/2 activation and leukemia cell proliferation.

Disclosures

No conflicts of interest to disclose.

Contributions

PPL, KFW and JNL performed experiments. PPL, KFW, JNL and PH designed the study, analyzed and interpreted the data. PPL and PH wrote the paper. KFW, MAO, WGW and PH aided in design of the microarray, processed its data. ZJX, MJK and YQL provided clinical samples and critical research materials. PPL, PH and YQL supervised the study.

Acknowledgements

The authors are most grateful to the Flow Cytometry Core Facility of Sun Yat-sen University Cancer Center for their technical support.

Funding

This work was supported by grants from the National Natural Science Foundation of China (grant no. 81972595), Natural Science Foundation of Guangdong Province (2023A1515010123) and a grant from the CLL Global Research Foundation.

Data-sharing statement

MicroRNA assay data has been made deposited in the Gene Expression Omnibus (GSE216258). All other datasets analyzed during the current study are available from the corresponding author on reasonable request.

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