There are several reports in the literature regarding microRNA (miR)-130b. It has been reported that miR-130b is involved in several diseases. The present study aimed to understand the association between the levels of miR-130b and lupus nephritis in patients. A total of 61 blood samples were collected and the expression level of miR-130b was determined. The online miRNA database was then searched using the ‘seed sequence’ located within the 3'-untranslated region of the target gene. Linear analysis and a luciferase assay were performed to understand the regulatory association between miR-130b and phosphatase and tensin homolog (PTEN). In addition, reverse transcription-polymerase chain reaction and western blot analyses were performed to examine the mRNA and protein expression levels of PTEN among individuals with lupus nephritis (n=28) and those without lupus nephritis (n=31), and in mesangial cells treated with scramble control, miR-130b mimics, PTEN small interfering (si)RNA and miR-130b inhibitors. In addition mesangial cells were treated with scramble control, miR-130b mimics, PTEN siRNA and miR-130b inhibitors to investigate the affect of miR-130b and PTEN on the viability and apoptosis of mesangial cells. The results demonstrated that miR-130b was downregulated in the hormone-resistant group of lupus nephritis patients. PTEN was a virtual target of miR-130b. There was a negative regulatory association between miR-130b and PTEN. The mRNA and protein expression levels of PTEN were increased in the hormone-resistant group. PTEN was a virtual target of miR-130b, and there was a negative regulatory association between miR-130b and PTEN. miR-130b and PTEN interfered with the viability and apoptosis of the mesangial cells.

Introduction

In total of ~0.1% of the population are affected by systemic lupus erythematosus (SLE), which is a chronic autoimmune disease involving multiple systems, with women having a nine times higher risk of developing the disease, compared with men (1). SLE is a complex disease, which has a range of manifestations and the combinations of which can lead to variable levels of disease severity. Autoreactive B and T lymphocytes have been the focus of the majority of studies performed to investigate the pathophysiology of this disease (2).

It is a general agreement that clinically relevant nephritis occurs in 60% of patients with lupus at some point during the duration of the disease (3). It is important that diagnosis is made early and that the treatment of renal disease occurs early, as early response to therapy is associated with more favorable results (4).

Current understanding of the correlation between gene messenger RNAs (mRNAs) and human disease has changed following the identification of microRNAs (miRNAs) at the turn of the 21st century, which marked a new era of cell biology and has extended to those sequences in the residual ~90% of eukaryotic genomes that produce non-coding RNAs (5). miRNAs function as meta-controls of gene expression and are pivotal for the cellular alterations required for development (5).

miRNAs are implicated in the pathogenesis of renal fibrosis and chronic kidney diseases. Certain miRNAs exhibit antifibrotic effects and others have profibrotic effects. miR-29 and miR-200 are reduced, whereas miR-21, miR-377, miR-205, miR-141 and miR-192 are elevated in animal models and patients with renal fibrosis, contributing to hypertensive nephrosclerosis, IgA nephropathy, obstructive nephropathy and diabetic nephropathy (6-10). Additionally, the suppression of miR-192 alleviates renal fibrosis in mice with diabetes (11), and it has been demonstrated that miRNAs serve as crucial mediators in renal fibrosis and may be promising targets for the prevention of end-stage renal disease (12). Few studies have focused on miRNAs in lupus nephritis. In a previous
study, 66 miRNAs differentially expressed in renal biopsies were identified from patients with lupus nephritis, compared with normal subjects. In another study, there were differences in the intrarenal expression levels of miR-146a, miR-198 and miR-638 between patients with lupus nephritis and normal subjects (13). There have been no reports on the potential of miRNAs as markers of any specific histologic presentation or their possible effect in renal fibrosis in lupus nephritis (14).

Patients with renal failure may have significantly decreased circulating miRNAs, compared with patients with marginal renal impairment or normal renal function, as shown by microarrays. The underlying mechanism may be associated with the substantial buildup of RNase in patients with renal failure, which elevates degraded circulating miRNAs, however, this remains to be elucidated (15). This was demonstrated by the fact that miR-1233-3p and miR-130b were downregulated, respectively, and that the total RNA level was significantly reduced in late stage lupus nephritis of the validation group, which were consistent with the results reported by Neal et al., who determined the serum levels of five specific miRNAs in patients who suffered from renal failure (16).

It has been previously shown that miR-130b is differentially expressed in lens epithelial cells collected from individuals with lupus nephritis, and the dysregulation of PTEN has also been reported to be involved in the molecular mechanism of mesangial cell apoptosis (17,18). By searching an online miRNA database, the present study identified PTEN as a potential target of miR-130b, and the involvement of miR-130b and PTEN in the development of lupus nephritis was confirmed.

Materials and methods

Sample collection. Tissue samples from 28 patients with SLE and lupus nephritis (33±4-years old) and 31 healthy patients (37±6-years-old) were collected between September 2013 and September 2014 at the Department of Rheumatism and Immunology, Tai’an Central Hospital (Shenyang, China). All patients were assessed by biopsy to confirm fulfillment to the 1982 American College of Hematology revised criteria for SLE and lupus nephritis (19). Written consent was signed by all patients, glomerular filtration rate was estimated using the modification of diet in renal disease formula (20), and clinical and demographic data of the patients were carefully recorded. The protocol of the present study was approved by the Ethics Committee of the Hospital of Tai’an Central Hospital.

Target prediction and functional analysis. By scanning the most commonly used target gene prediction databases, including miRDB (http://www.mirdb.org/), miRanda (http://www.microrna.org/microrna/home.do) and TargetScan (www.targetscan.org), the putative target genes of the miR-130b were pooled from the three databases in total. Experimental validation on most of the target genes was performed. Gene ontology (GO) term analysis were performed to gain a full insight of the functional relevance on these target genes. GO term analysis indicated that these target genes were enriched in critical biological processes, including ‘cell proliferation’ and ‘regulation of focal adhesion kinase activity’.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis of PTEN mRNA and miR-130b in tissue samples and mesangial cells. TRIzol extraction reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract the total RNA from tissue samples and mesangial cells (obtained from the tissue samples) according to manufacturer’s protocol. An A260/A280 value between 1.8 and 2.0 was accepted, which ensured that the RNA samples were without DNA or proteins. A TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to synthesize the cDNA (PTEN) from RNA with a mixture of 2 μg total RNA, 1 μg of miRNA-specific primers (25 μM) and ddH₂O (RNase-free) to a final volume of 10 μl. The mixture was then denatured for 10 min at 70˚C and placed on ice, followed by the addition of the reaction buffer comprising (11 μl ddH₂O (RNase-free), 4 μl dNTP mix, 4 μl 5X RT buffer and 1 μl RevertAse 100 U/μl; Toho Company, Ltd., Osaka, Japan), and then maintained for 60 min at 42˚C, followed by 10 min at 90˚C. In vitro Platinum SYBR-Green qPCR SuperMix-UDG (Thermo Fisher Scientific, Inc.) was used to amplify the cDNA according to the manufacturer’s protocol with a mixture of 1.5 μl forward primer (10 μl), 25 μl SYBR mix, 1.5 μl reverse primer (10 μM), 1 μl cDNA and 21 μl ddH₂O. Sequences of primers were: miR-130b forward, 5’GGCGAGTGAATGATTGAA3’, reverse, 5’GTGCGGTGTGTTGGAGTGC3’; PTEN forward, 5’-TTTGAGACATTACACCAACCCACAC-3’, reverse, 5’-ATTACACAGTTGCTGTCTTCTTTCC-3’ and GAPDH forward, 5’-AGCCTCAAGATCATCAGCAATG-3’ and reverse, 5’-TGTTGTCTAGTGATCTTCCACG-3’. Fluorescence detection and qPCR were performed using the Qiagen Rotor-Gene Q (Qiagen GmbH, Hilden, Germany). The reaction settings were as follows: 10 min at 50˚C, 10 min at 95˚C, 15 sec at 95˚C, 45 sec at 60˚C for 40 cycles. The 2^ΔΔCT method (21) was used to calculate the relative mRNA expression of PTEN and miR-130b according to the expression of GAPDH for human renal biopsies. Three assessments were performed in triplicate.

Cell culture and transfection. DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) was used for incubation of the mesangial cells. A TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to transfect the cells with miR-130b mimics or PTEN small interfering (si)RNA (Sunbio, Shanghai, China) or inhibitors according to protocols provided by the manufacturers. Sequence of PTEN siRNA was as follows: 5’-GGCGGAUAACAGGAAACAUAATT-3’. The medium was replaced with DMEM/F12 culture medium containing 2% reduced FBS 6 h following transfection. The experiments were repeated three times independently.

Cell proliferation assay. An MTT assay was used to assess the cell proliferation, as described previously. The mesangial cells were incubated in a 96-well plate at a final density of 5x10^4 cells per well for 1 day at 37˚C. Subsequently, 200 μl of MTT solution was used to treat the mesangial cells for 60 min at 37˚C, following which the MTT solution was carefully removed and
the cells were washed with 100 μl PBS. Finally, the cells were treated with 200 μl dimethyl sulfoxide solution for 120 min on a plate shaker at room temperature. A microplate reader (Synergy HT; BioTek, Instruments, Inc., Winooski, VT, USA) was used to measure the proliferation of the mesangial cells based on the absorbance at 575 nm. All tests were performed in triplicate.

**Luciferase assay.** The 3'-untranslated region (UTR) of PTEN containing the binding site of miR-130b was amplified by PCR and inserted into the psiCHECK-2 reporter vector (Promega Corporation, Madison, WI, USA). The mutagenesis was performed for the same site and introduced into the control vector (Ambion; thermo Fisher Scientific, Inc.). For transfection, the cells were co-transfected with wild-type/mutant type vector and miR-130b mimic/negative control using Lipofectamine® 2000 (thermo Fisher Scientific, Inc.) with a mixture of 1x10⁶ cells, 1 μg Repilla luciferase expression construct, PRL-TK (Promega Corporation), 1 μg of psiCHECK-2 reporter vector (Promega Corporation) or psiCHECK-2-mut plasmid and 50 pmol of miR-130b mimic or control. Renilla luciferase activity was used as an internal control. After incubation at 37°C for 48 h, the Dual Luciferase assay reagent (Promega Corporation) was used to measure the luciferase activity in accordance with the manufacturer's protocol. All results were calculated as fold differences relative to Renilla luciferase activity. Each experiment was performed at least three times.

**Western blot analysis.** For analysis of the mRNA expression of PTEN and miR-130b, ice-cold lysis buffer containing 1% NP-40, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl, including protease inhibitors (Roche Diagnostics, Basel, Switzerland) was used to lyse the mesangial cells according to the manufacturer's protocol. The lysates were centrifuged at 14,000 x g at 4°C for 15 min. A Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine the concentration of protein. To separate 35 μg protein, 8% SDS-PAGE was used, and the protein then was transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA) for 60 min (120 V). Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk was used to block the membrane to avoid unspecific binding. Primary antibodies, including anti-β-actin (1:5,000; A5441; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and anti-PTEN (1:1,000; SAB4300337; Sigma-Aldrich; Merck KGaA) were used to treat the membrane. Subsequently, HRP-conjugated secondary antibody (1:10,000; A6154; Sigma-Aldrich; Merck KGaA) was used for incubation of the membrane. Quantity One 1-D Analysis Software (v4.6.9; Bio-Rad Laboratories, Inc.) was used to quantify the band intensity.

**Analysis of apoptosis.** Apoptosis was performed using propidium iodide/annexin V staining with an apoptosis detection kit (Nanjing KeyGEN Biotech Co., Ltd., Nanjing, China). The mesangial cells were maintained at room temperature for 15 min in the dark, following which flow cytometry (BD Biosciences, San Jose, CA, USA) was used to assess the specimens. Annexin V immunofluorescence is shown on the X-axis and plasma membrane integrity is shown on the Y-axis. Analyses were repeated three times.

**Statistical analysis.** All data are shown as the mean ± standard error of the mean. Each experiment was performed at least three times to ensure the reproducibility of each test. One-way analysis of variance or Student's t-test was used to analyze differences to determine statistical significance between groups. Pearson's linear correlation analysis was used to analyze the correlation between two variables. The predictive accuracy of renal miR-130b levels was assessed using ROC analysis. P<0.05 was considered to indicate a statistically significant difference. SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was employed for statistical analysis. All tests were repeated three times.

**Results**

miR-130 is upregulated in patients with lupus nephritis. In the present study, kidney tissue samples were collected from patients with SLE. These samples were then divided into two groups based on whether they had nephritis or not: Lupus nephritis(+) and lupus nephritis(-), respectively. RT-qPCR analysis was then performed, and the results showed that the expression level of miR-130b was higher in the lupus nephritis(+) group (Fig. 1). These results indicated that miR-130b is a risk factor for lupus nephritis in patients.

PTEN is a virtual target of miR-130b. There is a series of literature on miR-130b, and it has been reported that miR-130b is involved in several diseases. The present study was performed to understand the association between miR-130b levels and lupus nephritis. An online miRNA target prediction tool was used to identify the regulatory gene of miR-130b, which identified PTEN as a candidate target gene of miR-130b with the 'seed sequence' in the 3'UTR (Fig. 2) and three existing binding sites. To confirm the regulatory association between miR-130b and PTEN, a luciferase activity reporter assay was performed in mesangial cells. Only the luciferase activity in the mesangial cells cotransfected with miR-130b and wild-type PTEN 3'UTR was decreased significantly (Fig. 3), whereas mesangial cells cotransfected with miR-130b and mutant PTEN 3'UTR showed comparable luciferase activity to that in the scramble control (Fig. 3). These results confirmed that PTEN was a target of miR-130b in mesangial cells. To further examine the regulatory association between miR-130b and PTEN, the correlation between the expression level of miR-130b and mRNA expression of PTEN was examined among the blood samples (n=61), which showed a negative regulatory association (Fig. 4; r=-0.5122; P<0.01).

**Determination of expression patterns of miR-130b and PTEN in tissues of different groups.** The tissue samples of the lupus nephritis(+) (n=28) and lupus nephritis(-) (n=33) patients were used to further examine the effects on the interaction between miR-130b and the PTEN 3'UTR. By performing RT-qPCR analysis, it was found that the mRNA expression of PTEN (Fig. 5A) was decreased in the lupus nephritis(+) group, compared with that in the lupus nephritis(-) group. The protein expression of PTEN (Fig. 5B) was measured using densitometric analysis, which revealed it was also decreased.
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in the lupus nephritis(+) group, compared with that in the lupus nephritis(-) group. To further confirm the hypothesis of the negative regulatory association between miR-130b and PTEN, the mesangial cells were transfected with scramble control, miR-130b mimics, PTEN siRNA and miR-130b inhibitors. The protein (Fig. 6A) and mRNA (Fig. 6B) expression levels of PTEN in the mesangial cells treated with iR-130b mimics and PTEN siRNA were lower, compared with those in the scramble control, whereas mesangial cells treated with miR-130b inhibitors were higher, compared with those in the scramble control. This confirmed the negative regulatory association between miR-130b and PTEN.

Figure 1. miR-130b is upregulated in patients with lupus nephritis. Expression of miR-130b was higher in the sensitive lupus nephritis(+) cells, compared with that in the resistant lupus nephritis(-) cells. miR, microRNA.

Figure 2. PTEN is a candidate target gene of miR-130b in mesangial cells. The ‘seed sequence’ was identified in the 3’untranslated region. There were three binding sites. miR, microRNA; PTEN, phosphatase and tensin homolog.

Figure 3. PTEN is a direct target of miR-130b. Luciferase activity reporter assay was performed to verify PTEN as the direct target gene of miR-130b. PTEN, phosphatase and tensin homolog; 3’UTR, 3’untranslated region; miR, microRNA.

Figure 4. Negative correlation between miR-130b and mRNA expression of PTEN. Correlation between the expression level of miR-130b and mRNA expression of PTEN between the lupus nephritis(+) group (n=28) and lupus nephritis(-) group (n=31). miR, microRNA; PTEN, phosphatase and tensin homolog.

Figure 5. Expression of PTEN is decreased in lupus nephritis. (A) mRNA and (B) protein expression levels of PTEN were decreased in the sensitive lupus nephritis(+) group, compared with those in the resistant lupus nephritis(-) group. PTEN, phosphatase and tensin homolog.
miR-130b and PTEN interfere with the viability of mesangial cells. The present study also investigated the relative viability of mesangial cells when transfected with scramble control, miR-130b mimics, PTEN siRNA and miR-130b inhibitors. The mesangial cells transfected with miR-130b inhibitors showed upregulated viability (Fig. 7A), compared with that in the scramble control, whereas the mesangial cells transfected with miR-130b mimics and PTEN siRNA showed comparably lower viability. This indicated that miR-130b negatively affected the viability of mesangial cells, whereas PTEN positively affected the viability of mesangial cells.

miR-130b and PTEN interfere with the apoptosis of mesangial cells. The present study subsequently investigated the relative apoptosis of mesangial cells when transfected with scramble control, miR-130b mimics, PTEN siRNA and miR-130b inhibitors. When transfected with miR-130b mimics and PTEN siRNA, the number of surviving mesangial cells was reduced and the number of apoptotic mesangial cells was increased, compared with numbers in the scramble control, whereas the mesangial cells transfected with miR-130b inhibitors showed comparably higher cell survival and reduced apoptosis (Fig. 7B). These results indicated that miR-130b accelerated apoptosis, whereas PTEN inhibited apoptosis.

Discussion

MiRNAs are highly conserved small noncoding RNAs involved in numerous biologic processes. MiR-130b was reported to be associated with numerous diseases, including type 2 diabetes (22), colorectal cancer (23), and bladder cancer (24). Zhaohui Ni revealed that patients with early stage LN exhibited a higher level of miR-130b compared with healthy patients, and suggested that miR-130b may be involved in LN by regulating the expression of Erbb2 interacting protein (17). Xiao Han demonstrated that miR-130b was associated with pathogenesis of LN via regulating IRF-1 and further inhibited the type I IFN pathway (25). It is important to further investigate the mechanisms underlying miR-130b, mesangial cells and proteinuria in the future. A carefully designed investigation performed on a larger sample size is also necessary and the inclusion of other kidney conditions is...
likely to provide more valuable evidence. In the present study, kidney tissue samples were collected from patients with SLE, which were divided into two groups based on whether they had nephritis or not: Lupus nephritis(+) and lupus nephritis(-). RT-qPCR analysis was performed, and the results showed that the expression level of miR-130b was higher in the lupus nephritis(+) group (Fig. 1). In addition, online miRNA target prediction tools were used to identify the regulatory gene of miR-130b, and PTEN was identified as the candidate target gene of miR-130b with the ‘seed sequence’ in the 3'UTR (Fig. 2) and the existence of binding sites. A luciferase activity reporter assay was also performed on the mesangial cells, in which luciferase activity was only reduced in the mesangial cells cotransfected with miR-130b and wild-type PTEN 3'UTR (Fig. 3). The luciferase activities in mesangial cells cotransfected with miR-130b and mutant PTEN 3'UTR were comparable with that of the scramble control (Fig. 3).

PTEN is a potent tumor-inhibitor gene present at chromosome 10q23.31, which was identified in 1997 (26,27). A phosphatase with double properties against proteins and phospholipids is encoded by PTEN (28). The signal transduction pathways can be regulated by PTEN protein via either phosphatase-independent or dependent mechanisms (29). Regardless of its possible serine/threonine and tyrosine phosphatase property, the tumor-inhibitory effect of PTEN contributes to its lipid phosphatase effect (30). PTEN is considered to be the major factor negatively regulating class I PI3Ks (31). Additionally, the regulatory effect triggered by specific miRNAs on the function of important immune cells, including B and T lymphocytes, in lupus has been investigated. PTEN regulates normal signaling via the B cell receptor, and abnormal miR-7 regulation results in hyper-responsiveness of B cells in SLE (32). In the present study, a microarray was performed to determine the expression levels of miRNAs in B cells obtained from patients with active SLE, compared with healthy subjects. A marked reduction in the expression of miR-1246 expression in B cells was found in patients with active SLE, but not in patients with inactive SLE or healthy subjects, which suggested that miR-1246 was involved in active SLE and may offer potential as a biomarker or promising therapeutic target in active SLE. In the present study, it was found that the miRNA expression of PTEN (Fig. 5A) was decreased in the lupus nephritis(+) group, compared with that in the lupus nephritis(-) group. The protein expression of PTEN (Fig. 5B) was also measured using densitometric analysis, which revealed its expression was decreased in lupus nephritis(+), compared with that in lupus nephritis(-), and that the expression level of PTEN in mesangial cells treated with miR-130b mimics and PTEN siRNA were lower, compared with that in the scramble control. The expression of PTEN in mesangial cells treated with miR-130b inhibitors was higher than that in the scramble control, confirming the negative regulatory association between miR-130b and PTEN.

Apoptosis can occur via mitochondrial and receptor-mediated pathways. The importance of apoptosis in SLE has been confirmed, as apoptotic cells result in a high level of autoantigens, which appear to be processed inappropriately in this disease (33). Apoptotic suppression with a pan-caspase inhibitor or with anti-Fas ligand antibodies results in prevention against renal disorder in models of lupus nephritis (34,35). It is increasingly accepted that inflammation and apoptosis can be correlated rather than being mutually exclusive (36). A previous observation that C3aRa inhibited apoptosis and inflammatory cell infiltration is consistent with this and may even be caused by the same mechanism (37). The first step toward its membrane correlation and complete activation is the phosphorylation of PKB/Akt on serine 473, which was found to be substantially elevated by the inhibition of C3aR, possibly leading to the apoptotic decrease observed (38). Additionally, significantly higher levels of phosphorylated PTEN (serine 380) have been observed in control lupus mice, compared with that in C3aRa-treated mice; as PTEN negatively regulates PI3K, and is closely associated with immune cell activation, cell growth and survival, these findings are likely to be correlated with the intrinsic pathophysiology of lupus nephritis and the mechanism by which signaling through C3aR causes disease (39,40). In the present study, the relative apoptosis was investigated when of mesangial cells were transfected with scramble control, miR-130b mimics, PTEN siRNA and miR-130b inhibitors. When transfected with miR-130b mimics and PTEN siRNA, the number of surviving mesangial cells was reduced and the number of apoptotic mesangial cells was increased, compared with those in the scramble controls group, whereas the mesangial cells transfected with miR-130b inhibitors showed comparably higher numbers of surviving cells and fewer apoptotic mesangial cells.

In conclusion, the present study demonstrated that miR-130b was upregulated in the lupus nephritis group, compared with that in the control group. PTEN was identified as a virtual target of miR-130b, and there was a negative regulatory association between miR-130b and PTEN. miR-130b and PTEN interfered with the viability and apoptosis of mesangial cells.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SW planned the study, collected the data, analysed and interpreted the data and prepared the manuscript. JW collected the data, prepared the manuscript. FL interpreted the data, collected the literature.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Hospital of Tai’an Central Hospital. Written consent was signed by all patients.
Consent for publication

Written consent was signed by all patients for the publication of any associated data or accompanying images.

Competing interests

The authors declare that they have no competing interests.

References