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Increased miR-374b promotes cell proliferation and the production of aberrant glycosylated IgA1 in B cells of IgA nephropathy

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ABSTRACT

The number of B cells is increased and the O-glycans of IgA1 are incompletely galactosylated in IgA nephropathy (IgAN). Here we report that expression of phosphatase and tensin homolog (PTEN) and Cosmc is decreased in B cells, and correlates with B cell number and the aberrant glycosylation of IgA1 in IgAN. Patients with IgAN exhibit higher miR-374b in B cells compared to controls. We show that miR-374b targets PTEN and Cosmc by luciferase assays and western blot analysis. Inhibition of miR-374b increased PTEN and Cosmc expression, and prevented cell proliferation and aberrant glycosylation of IgA1, thus representing a new therapeutic approach for IgAN.

1. Introduction

IgA nephropathy (IgAN), one of the most common forms of glomerulonephritis in the world, is characterized by the mesangial deposition of polymeric IgA1 within the kidney [1–3]. Increased percentages of B lymphocytes were noticed and especially in patients with increased concentration of immunoglobulin A in serum [4]. Phosphatase and tensin homolog (PTEN) inhibits phosphoinositide-3-kinase pathway by dephosphorylating PIP3 and prevents Akt activation [5]. Loss of PTEN in B cells of IgAN was evident in the array analysis conducted by Cox et al. [6], and decrease of PTEN has been found to be related to cell proliferation in various diseases [7,8].

On the other hand, serum IgA1 is abnormally O-glycosylated, which leads to mesangial deposition of IgA1 and the development of glomerular injury in IgAN [9]. Synthesis of O-glycans begins with the addition of N-acetylgalactosamine by the enzyme N-acetylga lactosaminyltransferase 2 and continues with the addition of galactose by the enzyme core 1 β1,3-galactosyltransferase (C1GALT1) [10]. Cosmc is required for the activity of the mammalian C1GALT1 [11]. Previous study indicates that Cosmc gene expression level is down regulated in the B cells of IgAN patients though the underlying mechanism is still unknown [12].

MiRNAs modulate protein expression by targeting mRNA transcripts and triggering either translation repression or RNA degradation [13]. Recent study shows that 37 miRNAs differentially expressed in PBMCs of patients with IgAN [14]. In the present study, blood B cells of IgAN are isolated and analyzed with miRNA chip array. Among the increased miRNA list, it is found that miR-374b may regulate the expression of both PTEN and Cosmc in B cells. The change of miR-374b in B cells of IgAN is confirmed, and the role of miR-374b in B cell proliferation and the production of aberrant glycosylation of IgA1 in IgAN are explored.

2. Materials and methods

2.1. Enrollment of patients

Thirty IgAN patients and 15 health controls were recruited for this study. Five in each group were included in array analysis, and other participants were used for PCR validation and in vitro experiment. The study was carried out in accordance with the principles of the Declaration of Helsinki under the approval of the ethics committees of Jinling Hospital.

2.2. Renal pathology

Percutaneous renal biopsy was performed in each patient under ultrasonographic guidance. Formalin-fixed renal tissue was

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embedded in paraffin using routine procedures. Thin 3 μm sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff, silver methenamine and Masson's trichrome [15]. Immunofluorescence staining was performed on 3 μm cryostat sections using FITC-labeled rabbit anti-human IgG, IgA, IgM, Complement (C) C3, C4 antibodies (Dako Corporation, Carpentaria, CA, USA). To evaluate kidney biopsy specimens using the IgAN Oxford classification [16], the following components were evaluated: mesangial proliferation (M) M1 or M0; endocapillary hypercellularity (E) E1 or E0; segmental sclerosis/adhesions (S) S1 or S0; tubular atrophy/interstitial fibrosis (T) T0 0–25%, T1 26–50%, or T2 >50%. The MEST score was calculated as the sum of M + E + S + T.

2.3. Flow cytometry

Blood cells were stained with FITC-conjugated, anti-CD19 mAb (Biolegend), then analyzed by flow-cytometer (Beckman Coulter, Fullerton, CA), acquiring 10000 events. The number of CD19+ B cells/μl was calculated from total lymphocytes per μl on the percentage of positive CD19 B cells determined by flow cytometry.

2.4. Isolation of peripheral B cells

B lymphoid cells were purified using anti-CD19 specific immunomagnetic beads according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

2.5. MicroRNA chip array analysis

Isolated B cells from five patients were pooled and extracted using miRCURY™ RNA Isolation Kits. Total RNA was labeled with miRCURY LNA microRNA Hy3 Power labeling kit (Cat #208031, Exiqon, Denmark), and analyzed with miRCURY LNA™ microRNA Array kit (Cat #208500, Exiqon, Denmark) according to the manual. The hybridization was carried out in accordance with the manufacturer's instructions. The slide was scanned with Genepix 4000B, and data analyzed with Genepix Pro 6.0. Signal intensities for each spot were scanned and calculated by subtracting local background. Normalization was performed through perchip 50th percentile method, allowing comparison among chips.

2.6. RT-PCR analysis

Template cDNA was prepared using reverse transcriptase, and miR-374b expression was quantified by SYBR® Premix Ex Taq™ II kit (TaKaRa, Dalin, China) with small nuclear RNA U6 as an endogenous control. Analysis of PTEN and Cosmc was performed using specific primers (PTEN sense, 5′-AATGGCTAAGTGAAGATGACAAT-3′, PTEN antisense, 5′-TGCACATATCATTACACCAGTTCGT-3′; Cosmc sense, 5′-AGTTTGCCTGAAATATGCTGGA-3′, Cosmc antisense, 5′-GGGGTGATAAGTCATTGCCTCT-3′).

2.7. Western blot analysis

Either 0.2–1 × 10^6 isolated blood B cells or DAKIKI cells were harvested and lyzed in RIPA buffer. Western blot analysis was performed as described previously [17], with antibodies against PTEN, Cosmc and β-actin purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.8. In situ hybridization analysis of miR-374b

Isolated B cells were fixed with 4% formaldehyde and centrifuged to slides by Cytospin. Cells were treated with 15 μg/ml proteinase K at 37 °C for 5 min. After washed, the slides were hybridized with 20 nM DIG-labeled miR-374b probe (Exiqon, Copenhagen, Denmark) diluted in hybridization buffer at 53 °C for 3 h. After washed, slides were treated with blocking buffer for 30 min, and then incubated with anti-DIG-AP in blocking buffer for 1 h. MiR-374b was visualized in a staining reaction with NBT/BCIP solution [18]. In all experiments, a negative control, staining without miR-374b probe, was performed.

2.9. Cells culture and treatment

Isolated blood B cells or DAKIKI cells were cultured with RPMI-1640 that was supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 25 mM HEPES buffer, and 10% heat-inactivated FBS [14]. Transfection of miRNA mimics or miRNA ASO was carried out by the TransIT-TKO Transfection Reagent (Mirus) according to the instruction. For knockdown of Cosmc, DAKIKI cells were transfected with siRNA, 5′-GCC UUU CUA UCU AGG CCA CAC UAU A-3′, with a concentration of 50 nM siRNA [19].

2.10. Luciferase assays

293T cells were transiently cotransfected with 0.1 μg of the reporter constructs (PTEN 3′-UTR and mutant PTEN 3′-UTR, or Cosmc 3′-UTR and mutant Cosmc 3′-UTR), 0.02 μg of the Renilla construct and 50 nM synthetic miR-374b mimics or control oligonucleotides. The firefly luciferase activity and Renilla luciferase activity were determined using the Dual-Luciferase Reporter Assay System (Promega). Values were normalized with Renilla luciferase [20,21].

2.11. B cell proliferation assay

Pure B lymphocytes were obtained by positive selection through magnetic-beads-conjugated anti-CD19 Ab and detached from beads according to the manufacturer's protocol. B cells were seeded in 96-well flat bottom plates. Cells were stimulated by CpG-ODN 2006 (6 μg/ml) for 5 days [22]. Proliferation was evaluated by BrdU Cell Proliferation ELISA Kit.

2.12. HAA lectin binding assays

The O-glycosylation profile of IgA1 was measured by binding of the *H. aspersa* lectin as previous report [23]. Briefly, 2.5 μg/ml of mouse anti-human IgA1 antibody was utilized to capture IgA1. The samples were adjusted to a final concentration of 1 μg/ml IgA1 in 1% BSA/PBST, and 100 μl of each sample was added to the reaction wells and then incubated at 4 °C overnight. The captured IgA1 was subsequently desialylated through treatment for 3 h at 37 °C with 20 mU/ml neuraminidase. After washes, 2 μg/ml of biotinylated *H. aspersa* lectin was added to the reaction wells, and incubated for 3 h at 37 °C. Lectin binding was detected with avidin–horseradish peroxidase conjugate, and the reaction was developed with the peroxidase chromogenic substrate o-phenylenediamine–H_2O_2 (SIGMA). The color reaction was ceased with 2 N H_2SO_4, and the optical density in duplicate samples at 490 nm was determined in a microplate reader.

2.13. Statistical analyses

Data is expressed as mean ± SD or percentage. Statistical analysis of the data from multiple groups was performed by one-way ANOVA followed by Student–Newman–Kuels test. The data from two groups was compared through *t* test. Linear regression was applied to model the relationship between the level of miR-374b