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*Original Article*

# Role of let-7b in the regulation of *N*-acetylgalactosaminyltransferase 2 in IgA nephropathy

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## ABSTRACT

**Background.** IgA nephropathy (IgAN) is characterized by aberrant O-glycosylation in the hinge region of IgA1. The early step in O-glycan formation is the attachment of *N*-acetylgalactosamine (GalNAc) to the serine/threonine of the hinge region; the process is catalysed by UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 2 (GALNT2). In our previous work, the microarray analysis on peripheral blood mononuclear cells (PBMCs) identified an upregulated miRNA called let-7b.

**Methods.** To study the molecular mechanisms in which let-7b was involved, we performed a bioinformatic analysis to predict their target genes. To validate biologically let-7b targets, we performed transient transfection experiments *ex vivo* using PBMCs from an independent group of IgAN patients and healthy blood donors (HBDs).

**Results.** Bioinformatic analysis revealed that GALNT2 is the potential target of let-7b. We found this miRNA significantly upregulated in PBMCs of IgAN patients compared with HBDs. Then, we demonstrated in *ex-vivo* experiments that let-7b decreased GALNT2 levels in PBMCs of IgAN patients, whereas the loss of let-7b function in PBMCs of HBDs led to an increase of GALNT2 mRNA and its protein level. Finally, we found that upregulation of let-7b occurred also in B-lymphocytes from IgAN patients.

**Conclusions.** Our results give novel additional information on the abnormal O-glycosylation process of IgA1 in IgAN patients. This study provides evidence for another important miRNA-based regulatory mechanism of the O-glycosylation process in which the deregulated expression of let-7b is associated with altered expression of GALNT2. This finding could be taken into consideration for new therapeutic approaches in IgAN because other serum glycosylated proteins do not display abnormal glycosylation.

**Keywords:** Iga nephropathy, microRNAs, O-glycosylation process, peripheral blood mononuclear cells, UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 2 (GALNT2)

## INTRODUCTION

Immunoglobulin A nephropathy (IgAN) is the most common biopsy-proven primary glomerulonephritis worldwide [1]. This disease is characterized by the dominance or codominance of IgA1 in the mesangial immune deposits that originate from circulating IgA1-containing immune complexes and/or polymeric IgA1 [2–4].

The human IgA1 molecule in serum contains, in its hinge region, multiple O-linked oligosaccharides (O-glycans) [5] that are assembled by the stepwise addition of monosaccharides (Supplementary Figure S1). It is initiated by the addition of *N*-acetylgalactosamine (GalNAc) to serine or threonine residues through the activity of the UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 2 (GALNT2) [6]. Thereafter, galactose is attached to GalNAc by core 1 β1,3-galactosyltransferase (C1GALT1) [7, 8] in association

with its chaperone, Cosmc [9]. Sialic acid, or *N*-acetylneuraminic acid (NeuAc), may be linked to GalNAc by α2,6-GalNAc-sialyltransferase I or II (ST6GalNAcI or ST6GalNAcII) [10]. NeuAc can also be attached to galactose by α2,3-Gal-sialyltransferase (ST3Gal) [11].

Although the aetiology of IgAN remains unclear, aberrant O-glycosylation in the hinge portion of serum IgA1 is thought to be deeply implicated in the pathogenesis [4, 12]. The mechanisms concerning the formation of the aberrant glycans are not well understood, but likely include changes in the expression and/or activity of enzymes involved in the individual glycosylation steps.

In our recent study, we studied the microRNA (miRNA) expression profile in peripheral blood mononuclear cells (PBMCs) of IgAN patients [13]. We provided evidence for a novel pathological mechanism whereby a miRNA, called miR-148b, regulates the levels of C1GALT1 gene and protein expression suggesting a key role of this miRNA in the regulation of the glycosylation process in IgAN.

In the current study, we continued our investigation of the functional role of miRNAs found up-regulated in our previous work [13], in particular let-7b. We demonstrated that let-7b was significantly up-regulated in IgAN patients and this miRNA modulated the enzyme GALNT2 expression that participates in early phase of the glycosylation process.

ORIGINAL ARTICLE

## MATERIALS AND METHODS

### Patients

A total of 76 biopsy-proven IgAN patients, 76 healthy blood donors (HBDs) and 8 disease controls (patients with membranoproliferative glomerulonephritis and focal segmental glomerulosclerosis) were included in this study. Initially, we selected 58 IgAN patients and 58 HBDs samples for let-7b real-time PCR. From each group, we used 15 samples for GALNT2 real-time PCR and 10 samples for GALNT2 protein expression. These groups overlapped with that of our previous study [13]. An independent cohort of 18 IgAN patients and 18 HBDs were used for the *in vitro* transfections and for the $CD19^+$ isolation.

All participants included in the study were from the South of Italy. The study was carried out according to the principles of the Declaration of Helsinki and was approved by our institutional ethics review board. Written informed consent was obtained from each patient and HBDs.

The HBDs enrolled in the study were selected on the basis of their demographical characteristics and overlapped completely with IgAN group. All HBDs included in this study had a negative urine test for blood and proteins.

All patients suffering from diabetes, chronic lung diseases, neoplasm, or inflammatory diseases and patients receiving antibiotics, corticosteroids and nonsteroidal anti-inflammatory agents were excluded from the study. No patients had symptomatic coronary artery diseases or a family history of premature cardiovascular diseases.

The main demographic and clinical features of our patients and controls included in the study are summarized in Table 1.

**Table 1. Demographic and clinical features of all patients and healthy blood donors (HBDs) included in the study**

| | IgAN | HBDs | non-IgAN |
|---|---|---|---|
| Number | 76 | 76 | 8 |
| Gender (M/F) | 55/21 | 52/24 | 5/3 |
| Age (Years) | 36 ± 12 | 42.3 ± 10.8 | 42 ± 20.5 |
| sCr (mg/dL) | 1.5 ± 1.2 | nd | 0.8 ± 0.4 |
| eGFR | 91.4 ± 44.2 | nd | 116.8 ± 10.5 |
| Systolic BP (mmHg) | 125 ± 15.6 | 122 ± 16.8 | 126 ± 14.9 |
| Diastolic BP (mmHg) | 76 ± 8.1 | 75 ± 8.5 | 78.6 ± 6.9 |
| Histological classification (G1/G2/G3) | 22/31/8 | n.d | n.d |

IgAN, IgA nephropathy, sCr, serum creatinine; eGFR, estimated glomerular filtration rate (eGFR has been calculated using the CKD-EPI creatinine formula, mL/min per 1.73 $m^2$); nd, not determined.
Values are expressed as mean ± SD.

There were no statistically significant differences between IgAN patients and HBDs for all parameters considered.

### PBMCs and B-lymphocyte subset isolation

PBMCs were isolated by density separation over a Ficoll–Hypaque (Ficoll-Paque Plus, GE Healthcare) gradient (460 g for 30 min). PBMCs were washed three times with PBS pH 7.4/1 mM EDTA (Sigma). Cells were then counted and their viability was determined by Trypan blue exclusion. B-lymphocytes were obtained from PBMCs by immunolabelling the sample with CD19-positive selection kit (EasySep, StemCell Technologies) according to the manufacturer's specifications. The purity of positively selected samples was typically >95% $CD19^+$ by flow cytometric analysis (data not shown).

### miRNA isolation

PBMCs and B-lymphocytes isolated from IgAN patients and HBDs were used for total RNA extraction by means of miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. DNase treatment was carried out to remove any contaminating DNA (RNase-Free DNase Set, Qiagen).

Total RNA, including small RNA fractions, was then eluted in RNase-free water. The RNA concentration was determined with NanoDrop Spectrophotometer (Nanodrop Technologies).

### miRNA target gene prediction

miRNA targets were predicted by means of miRBase 19.0 (http://microrna.sanger.ac.uk, [14]), TargetScan 5.1 (http://www.targetscan.org/, [15]), PicTar (http://pictar.org, [16]) and RNA22 1.0 (http://cm.jefferson.edu/rna22v1.0, [17]) algorithms. Potential targets were chosen overlapping results from the four algorithms and selecting gene targets predicted by at least two of them and based on a score cut-off computed by a weighted sum of a number of sequence and context features of the predicted miRNA:mRNA duplex.

### Quantitative RT–PCR

Total RNA, including small RNA fractions, was reverse transcribed with miScript Reverse Transcription Kit (Qiagen) following the manufacturer's instructions.

The real-time reverse-transcription polymerase chain reaction (RT–PCR) for the quantification of a let-7b plus two selected endogenous control was carried out with miScript Primer Assays and miScript SYBR Green PCR Kit from Qiagen. Real-time PCR amplification reactions were performed in triplicate in 25 µL of final volume via SYBR Green chemistry on iCycler (Bio-Rad).

Two normalization steps were used: the first step was performed normalizing the data against a small nucleolar RNA U6 (endogenous control commonly used as normalizer) and the second one using the miR-27a, a microarray internal control highly and equally expressed in our microarray samples [13].

Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the $2^{-\Delta Ct}$ method.

For the GALNT2 expression analysis, quantitative RT–PCR amplification reactions were performed in triplicate in 25 µL final volumes using SYBR Green chemistry on an iCycler. Quantitative RT–PCR was performed using the QuantiTect Primer Assay and the QuantiFast SYBR Green PCR mix (Qiagen). Genes were amplified according to the manufacturer's directions. The β-actin gene amplification was used as a reference standard to normalize the target signal.

### Let-7b mimic and inhibitor transfection

Isolated PBMCs were cultured in a 12-well plate with RPMI-1640 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.

The transfection of miRNAs mimic and inhibitor was carried out using TransIT-TKO Transfection Reagent (Mirus) in accordance with the manufacturer's procedure. Let-7b mimic and let-7b inhibitor were purchased from Qiagen. In transfection experiments a mock-transfection control was performed by putting cells through the transfection procedure without adding miRNA. The validated nonsilencing siRNA sequence AllStars Negative Control siRNA (50 nM, Qiagen) was used as negative control and Syn-hsa-miR-1 miScript miRNA Mimic (25 nM, Qiagen) and Anti-hsa-miR-1 miScript miRNA Inhibitor (400 nM, Qiagen) were used as positive controls for let-7b mimic and let-7b inhibitor transfection, respectively. Each transfection experiment was done in triplicate.

After transfection, cells were incubated at 37°C for 24 or 72 h and used, respectively, for total RNA extraction and protein extraction.

### Western blot

The amount of GALNT2, after transfection, was determined by western blotting analysis. Total protein extracts were prepared with RIPA lysis buffer containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA, 1.5% NonidetP-40, 1 mM sodium orthovanadate, plus proteinase inhibitors. The protein concentration was determined by the Bradford assay (BioRad). Fifty micrograms of each protein lysate was separated on a 10% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were incubated in 5% non-fat milk powder diluted in PBS containing 0.1% Tween-20 (T-PBS) for 2 h at room temperature (RT) and probed with a monoclonal anti-GALNT2 antibody (Abcam Biologicals) in blocking buffer overnight at 4°C. Finally, membranes were incubated with secondary antibody of horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz). Immunocomplexes were detected with the ECL method (GE Healthcare, Amersham, UK). The same membranes were stripped and reprobed with anti-actin monoclonal antibody (Sigma).

Images of autoradiography were acquired using a scanner EPSON Perfection 2580 Photo (EPSON) and quantified by Image J 1.34 Software (http://rsb.info.nih.gov/ij/). Ratio between intensities of GALNT2 and β-actin bands was used to normalize GALNT2 expression in each sample.

### Statistical analysis

Two-tailed Student's *t*-test was used to assess differences in biological features among IgAN patients and HBDs. Pearson's correlation test was used to study continuous variables. All values were expressed as the mean ± SEM of data obtained from at least three independent experiments. Results were considered statistically significant at $P < 0.05$.

## RESULTS

### Validation of let-7b upregulation

In our previous work, we reported that let-7b is one of the most upregulated miRNAs in IgAN patients [13]. In order to further validate the let-7b upregulation, we performed quantitative real-time PCR (q-RT–PCR) for let-7b on miRNAs isolated from PBMCs of 58 IgAN patients and 58 healthy blood donors (HBDs). The expression of let-7b was significantly higher in IgAN patients compared with HBDs ($P < 0.0001$; Figure 1). Then, we moved on to investigate whether let-7b upregulation was specific of IgAN. We compared the previously let-7b levels of IgAN patients and HBDs with let-7b expression

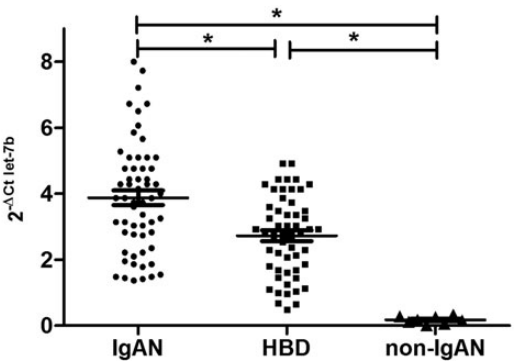
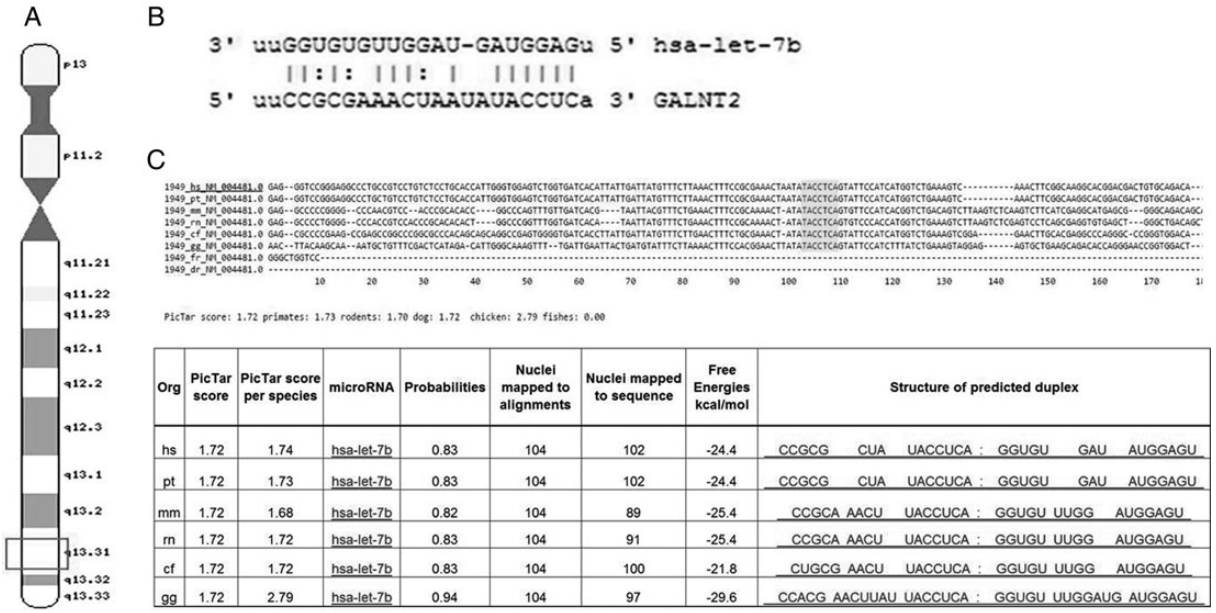



FIGURE 1: Validation of differential expression of let-7b in PBMCs of 58 IgAN patients, 58 healthy blood donors (HBDs) and 8 non-IgAN patients. Expression levels were quantified using the quantitative real-time PCR (q-RT–PCR). The miRNA relative expressions were normalized to the expression of miR-27a and of U6. Expression levels of let-7b were found significantly higher in IgAN patients compared with HBDs and patients with other glomerulonephritides. The histograms represent the mean ± SEM. *$P < 0.0001$.




| Org | PicTar score | PicTar score per species | microRNA | Probabilities | Nuclei mapped to alignments | Nuclei mapped to sequence | Free Energies kcal/mol | Structure of predicted duplex |
|---|---|---|---|---|---|---|---|---|
| hs | 1.72 | 1.74 | hsa-let-7b | 0.83 | 104 | 102 | -24.4 | CCGCG CUA UACCUCA : GGUGU GAU AUGGAGU |
| pt | 1.72 | 1.73 | hsa-let-7b | 0.83 | 104 | 102 | -24.4 | CCGCG CUA UACCUCA : GGUGU GAU AUGGAGU |
| mm | 1.72 | 1.68 | hsa-let-7b | 0.82 | 104 | 89 | -25.4 | CCGCA AACU UACCUCA : GGUGU UUGG AUGGAGU |
| rn | 1.72 | 1.72 | hsa-let-7b | 0.83 | 104 | 91 | -25.4 | CCGCA AACU UACCUCA : GGUGU UUGG AUGGAGU |
| cf | 1.72 | 1.72 | hsa-let-7b | 0.83 | 104 | 100 | -21.8 | CUGCG AACU UACCUCA : GGUGU UUGG AUGGAGU |
| gg | 1.72 | 2.79 | hsa-let-7b | 0.94 | 104 | 97 | -29.6 | CCACG AACUUAU UACCUCA : GGUGU UUGGAUG AUGGAGU |

**FIGURE 2:** Let-7b targets GALNT2. (**A**) Genomic localization of let-7b (chromosome 22q13.31). (**B**) let-7b binding site at 3057–3078 nt of the GALNT2 3′UTR. (**C**) Sequence alignment of the let-7b base-pairing sites in the 3′-UTR of GALNT2 mRNA showing that the regions complementary to let-7b are highly conserved among the human, chimp, mouse, rat, dog and chicken. The 'seed' sequences of let-7b complementary to GALNT2 are shown in gray.

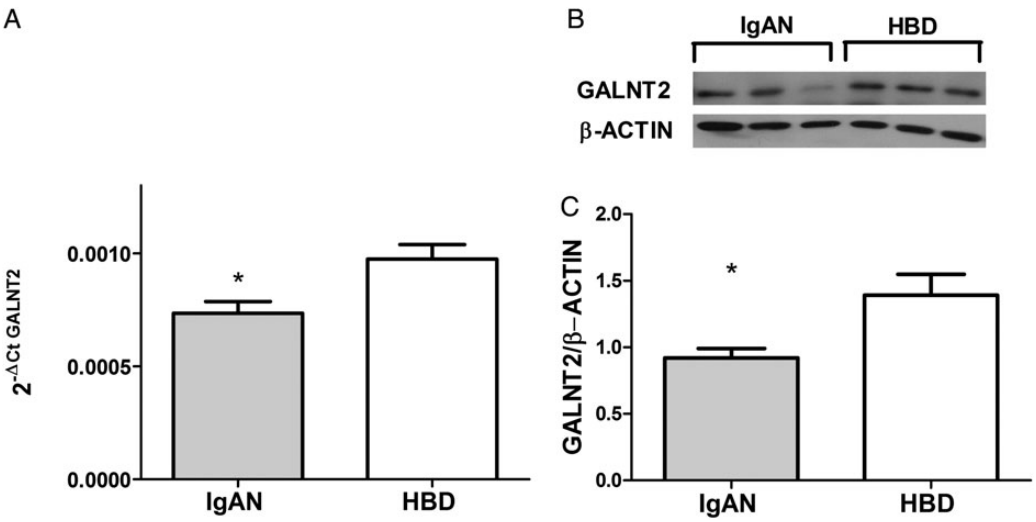



**FIGURE 3:** GALNT2 expression in IgAN patients. (**A**) GALNT2 gene expression levels evaluated by real-time PCR in 15 IgAN patients and 15 healthy blood donors (HBDs). GALNT2 expression levels were found significantly lower in IgAN patients compared with HBDs. GALNT2 expression levels were normalized on the housekeeping gene β-actin. The histograms represent the mean ± SEM. *P = 0.008. (**B**) Representative western blotting experiment for GALNT2. (**C**) GALNT2 protein levels of 10 IgAN patients and 10 HBDs evaluated by western blot. In accordance with mRNA expression levels, GALNT2 protein levels were significantly lower in IgAN patients compared with HBDs (P = 0.01).

in PBMCs from patients with other glomerular diseases. We found that let-7b expression levels were again higher in IgAN patients ($P < 0.0001$) compared with disease controls (Figure 1) confirming that higher let-7b levels are specific of IgAN.

### *In silico* analysis of let-7b targets

Let-7b is encoded by the MIRLET7B gene located in the chromosome 22q13.31 (Figure 2A) and expressed in 32 species (miRBase Sequence Database, http://www.mirbase.org, 14). To study the molecular mechanisms in which let-7b is involved, we performed a bioinformatic analysis to predict potential let-7b target genes. To reduce the number of false positives, we used four different algorithms and we listed only putative target genes predicted by at least two of them (Supplementary Table S1). Based on the results of bioinformatics analysis we found that one of the potential targets of let-7b was the gene UDP-*N*-Acetyl-Alpha-D-Galactosamine:polypeptide

*N*-acetylgalactosaminyltransferase 2 (GALNT2) that plays an essential role in the pathogenesis of IgAN; in fact, this gene is involved in the abnormal glycosylation processes of IgA1 in IgAN [6]. Moreover, our alignment studies within GALNT2 mRNA sequences from multiple organisms confirmed a highly conserved binding site for let-7b within the GALNT2 3′UTR (Figure 2B and C). Interestingly, let-7b seems to regulate other isoforms of *N*-acetylgalactosaminyltransferase as GALNT1, -4, -6, -7, -10, -11, -13.

### GALNT2 expression in IgAN patients

It has been speculated that GALNT2 is responsible for initiation of O-glycosylation in IgA1. However, GALNT2 expression in IgAN has been little investigated. Therefore, we evaluated its mRNA expression by real-time PCR (qRT–PCR) in PBMCs of 15 IgAN patients and 15 HBDs. We found that GALNT2 levels were significantly lower in IgAN patients compared with HBDs (P = 0.008; Figure 3A).

To study whether down-regulation of GALNT2 in IgAN patients led to protein reduction, we measured the protein levels of GALNT2 in PBMC lysate of 10 IgAN patients and 10 HBDs. The GALNT2 protein levels were significantly lower in IgAN patients (0.9 ± 0.07 GALNT2/β-actin ratio) compared with HBDs (1.4 ± 0.15 GALNT2/β-actin ratio) (P = 0.01; Figure 3B and C).

To study whether the down-regulation of GALNT2 in IgAN patients was attributable to increased let-7b levels, we tested the correlation between GALNT2 mRNA and let-7b levels in IgAN patients and HBDs. A negative correlation was observed ($r = -0.412$, $P < 0.02$; Figure 4); in fact, samples with higher let-7b expression levels showed lower levels of GALNT2 mRNA.

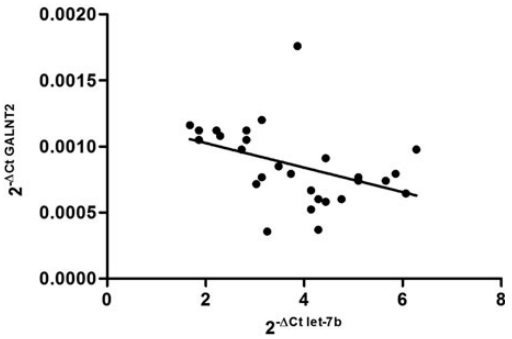



**FIGURE 4:** Inverse correlation between let-7b and GALNT2 expression in IgAN patients and healthy blood donors (HBDs). Expression of GALNT2 analysed by qRT–PCR and normalized to β-actin. The let-7b expression was examined by qRT–PCR analysis and normalized to the expression of miR-27a and of U6. Statistical analysis was performed using Pearson's correlation coefficient ($r = -0.412$, *P = 0.02).

### Let-7b directly decreases GALNT2 mRNA expression

Our *in silico* analysis showed that the let-7b increase could be the cause of the GALNT2 reduction. To test whether the let-7b is able to modulate the expression of the GALNT2 mRNA, we performed transient transfection experiments *ex vivo* using PBMCs from four IgAN patients and four HBDs. We increased the amount of the endogenous let-7b within PBMCs from HBDs, transfecting short RNA sequences that mimic the action of the miRNA, in order to simulate the situation found in IgAN patients. PBMCs from HBDs, transfected with 25 nM let-7b mimic and incubated for 24 h, showed a reduction (P = 0.04) of endogenous GALNT2 mRNA levels (Figure 5A).

In the reverse experiment, to further confirm our data, we used synthetic hairpin miRNA inhibitors to silence the activity of let-7b in PBMCs isolated from IgAN patients and evaluated the expression of GALNT2. The transiently transfection of IgAN PBMCs with 400 nM let-7b inhibitor led to an increase (P = 0.03) of endogenous GALNT2 mRNA levels (Figure 5B).

### Let-7b decreases GALNT2 protein expression

We used the same strategy to functionally inhibit or to enhance the mature form of let-7b in PBMCs of IgAN patients or HBDs, respectively, with the aim of establishing whether also GALNT2 protein expression was effectively controlled by

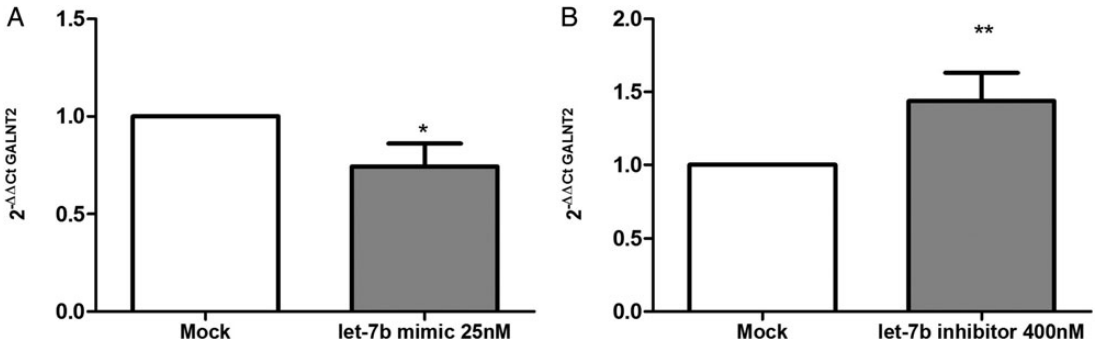



**FIGURE 5:** Modulation of GALNT2 mRNA in PBMCs by let-7b. (**A**) GALNT2 expression levels were analysed by real-time PCR in PBMCs of healthy blood donors (HBDs) following transfection with let-7b mimic. Rising endogenous let-7b within HBDs PBMCs resulted in a reduction of endogenous GALNT2 mRNA levels. (**B**) GALNT2 expression levels were analysed by real-time PCR in PBMCs of IgAN patients following transfection with let-7b inhibitor. Silencing the activity of let-7b within IgAN PBMCs led to an increase of endogenous GALNT2 mRNA levels. Mock indicates mock-transfected cells going through the transfection processes without addition of mimic/inhibitor miRNA. Expression data were normalized on the housekeeping gene β-actin. Data are representative of four independent experiments (means ± SEM), *P = 0.04; **P = 0.03.

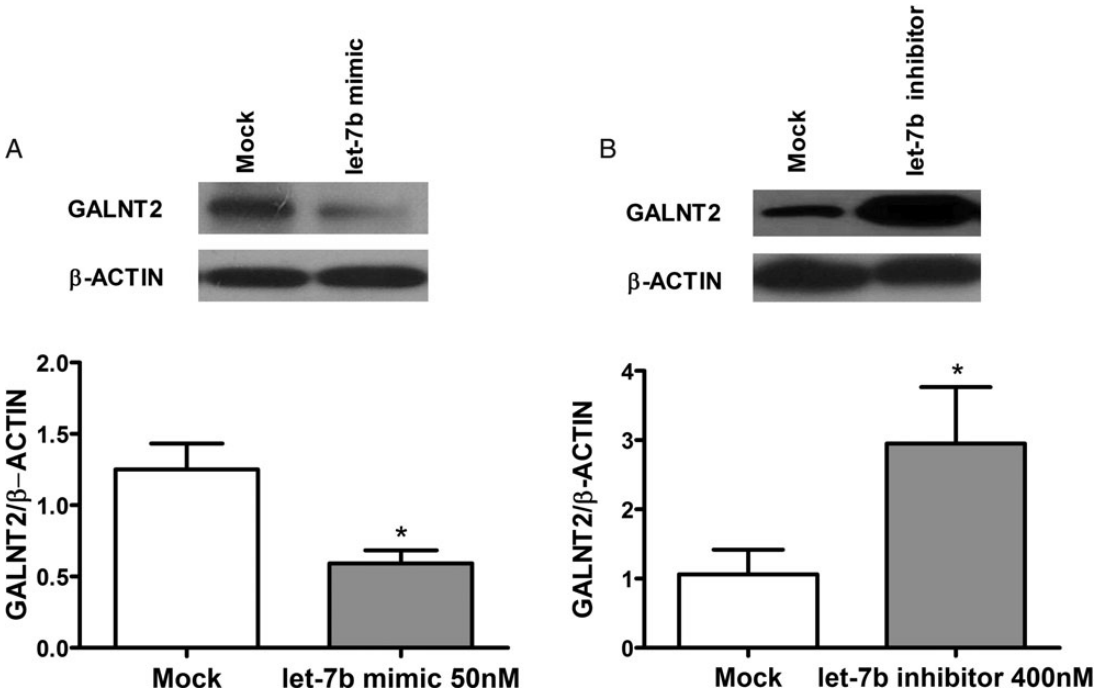



**FIGURE 6:** Modulation of GALNT2 protein expression in PBMCs by let-7b. (**A**) Transfection of PBMCs of healthy blood donors (HBDs) with 50 nM let-7b mimic resulted in a 2.1-fold reduction of GALNT2 protein expression. (**B**) Western blot representing protein levels of GALNT2 in IgAN PBMCs following transfection with 400 nM let-7b inhibitor. A significant increase in GALNT2 protein production was shown in IgAN PBMCs transfected with let-7b inhibitor (2.8-fold increase). Mock indicates mock-transfected cells going through the transfection processes without addition of mimic/inhibitor miRNA. In both experiments, β-actin was used as endogenous control. Data are representative of four independent experiments (means ± SEM). *P = 0.02.

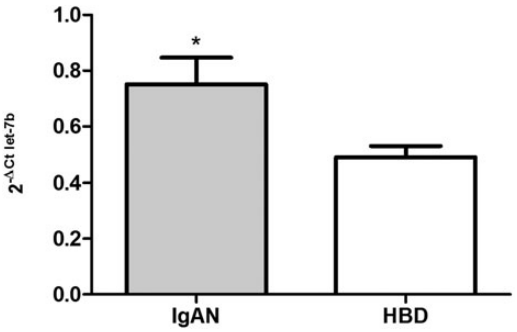



**FIGURE 7:** Let-7b in B lymphocytes from 10 IgAN patients and 10 healthy blood donors (HBDs). Expression levels were quantified using the quantitative real-time PCR (q-RT–PCR). The miRNA relative expressions were normalized to the expression of miR-27a and of U6. We found that expression levels of let-7b were significantly higher in IgAN patients compared with HBDs. The histograms represent the mean ± SEM. *P < 0.01.

let-7b. PBMCs from four HBDs were transiently transfected with 50 nM let-7b mimic, and incubated for 72 h. GALNT2 protein expression was determined by means of western blot. Protein analysis revealed that in normal PBMCs, transfection of let-7b mimic resulted in a remarkable reduction (2.1-fold, P = 0.02; Figure 6A) of GALNT2 protein expression. In the reverse experiment, PBMCs from four IgAN patients transfected with 400 nM let-7b inhibitor showed a significant increase of GALNT2 protein production (2.8-fold increase, P = 0.02; Figure 6B).

All together our results demonstrated that let-7b targets GALNT2, responsible for a decreased GALNT2 expression in IgAN.

### Let-7b expression in B-lymphocytes from IgAN patients

Since IgA1 glycosylation occurs within B lymphocytes, we performed quantitative real-time PCR (q-RT–PCR) for let-7b on miRNAs isolated from B-lymphocytes ($CD19^+$) of an independent group of 10 IgAN patients and 10 HBDs. The expression of let-7b was significantly higher in B-lymphocytes of IgAN patients compared with HBDs (P < 0.01; Figure 7).

## DISCUSSION

Aberrant glycosylation of serum IgA1 plays an important role in the pathogenesis of IgAN. It is apparent that there is over-representation of IgA1 molecules with certain O-glycan patterns in the serum of patients with IgAN and that these O-glycoforms predominate in the mesangial deposits [18]. This IgA1 abnormality might be synthetic rather than degradative, since other serum proteins with O-glycosylation do not display abnormal glycosylation [19].

The O-glycan chains of IgA1 are formed by the sequential reactions that are catalysed by some glycosyltransferases. In the last years, more studies have focused attention on the altered expression or activity of glycosyltransferases involved in the pathogenesis of IgAN (Supplementary Figure S1) [20–24].

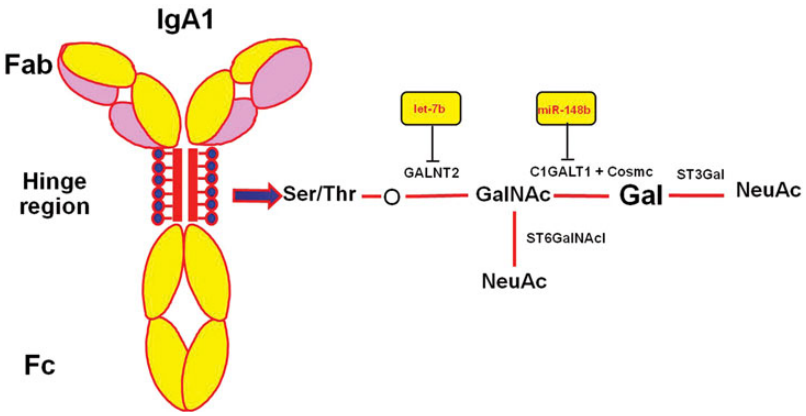



**FIGURE 8:** Schematic representation of how let-7b and miR-148b might modulate the IgA1 O-glycosylation process.

It has been suggested that core 1 β1,3-galactosyltransferase (C1GALT1), the intracellular enzyme that catalyses the addition of galactose to O-linked GalNAc moieties, is reduced in patients with IgAN [24, 25]. Moreover, in our recent paper we reported an important role of miRNA in the pathogenesis of IgAN; in particular we demonstrated that lower levels of C1GALT1 due to inhibiting effect of over-expressed miR-148b, may lead to an increase of circulating deglycosylated IgA1 [13].

However, the UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 2 (GALNT2), as one of the other important glycosyltransferases in IgAN, has been rarely documented. In the current study, we detected that the expression of GALNT2 at mRNA and protein level; we found that the expression of GALNT2 mRNA and protein were decreased in PBMCs of patients with IgAN. This was consistent with another previous study that investigated the expression of GALNT2 in tonsillar B lymphocytes of IgAN patients [26]. Furthermore, previous studies have quantitatively analysed the O-glycan structure in the serum IgA1 hinge region using mass spectrometry (MS) and matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOFMS) showing that not only the number of Gal was reduced in IgAN patients but also GalNAc content [18, 27].

Starting from microarray miRNA expression profile in PBMCs of IgAN patients published in our recent paper, in the current study we focused our attention on let-7b, the most upregulated miRNA in IgAN patients, and their target genes. Among many putative target genes identified by bioinformatic analysis, GALNT2 aroused our interest because it could be involved in the abnormal glycosylation process of IgA1 in IgAN [13].

In support of our bioinformatic analysis, we found a reduced expression of GALNT2 in IgAN patients and we showed that let-7b expression negatively correlated with the GALNT2 expression levels in the same subjects. Moreover, using the *ex-vivo* strategy to functionally enhance or inhibit the mature form of let-7b, we confirmed biologically that let-7b targets GALNT2 and is able to modulate its mRNA and protein levels. An additional investigation of this data was carried out on B lymphocytes, wherein we identified high levels of let-7b expression in IgAN patients. Thus, we supposed that let-7b could regulate GALNT2 also in B-lymphocytes.

Our data on GALNT2 regulation by let-7b give reason for an additional mechanism that regulates the first reaction of IgA1 O-glycosylation in IgAN (Figure 8). Indeed, we hypothesized that there is a synergistic mechanism between let-7b and miR-148b in the regulation of IgA1 O-glycosylation since we observed a direct correlation between the two miRNAs; in fact subjects with high levels of let-7b had high levels of miR-148b (Supplementary Figure S2). Future studies will be needed to demonstrate whether the reduction of the enzyme GALNT2 causes a reduction of GalNAc content in the hinge region of IgA1.

Our study has two limitations. First, we did not study the effect of let-7b on the process of IgA1 glycosylation in an experimental animal model of IgAN because IgA1 is present exclusively in humans and hominoid primates [28, 29]. Second, the study was limited to participants of Caucasian ancestry.

In summary, this study provides evidence for another important miRNA-based regulatory mechanism of the O-glycosylation process in IgAN discovering that high expression of let-7b is associated with low expression of GALNT2. These findings could also have potential therapeutic implications, since the let-7b levels may be manipulated to provide new therapeutic approaches to the disease.

## SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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## AUTHOR CONTRIBUTIONS

G.S. and F.P.S. designed the experiments; G.S., F.S., C.C., S.N.C. and G.D.P. performed the experiments; F.P. and F.P.S. collected blood samples and furnished clinical and laboratory data; G.S. and F.S. analysed data; G.S. and F.P.S. wrote the paper.

## CONFLICT OF INTEREST STATEMENT

None declared.

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