PREPARAZIONE DI UN KIT DIAGNOSTICO COMMERCIALE PER IL DOSAGGIO SIERICO DEL miR-148b NELLA MALATTIA RENALE: IMMUNOGLOBULINA A NEFROPATIA (IgAN)

F.P. Schena

Università degli Studi di Bari; Consorzio C.A.R.S.O. (Centro Addestramento Ricerca Scientifica in Oncologia) - Valenzano (Bari)
Immunoglobulin A nephropathy (IgAN) is a worldwide kidney disease characterized by recurrent episodes of gross hematuria (red/coke colour) in concomitance of upper respiratory tract infections or by permanent microhematuria and/or proteinuria.
IgA nephropathy (IgAN) is characterized by aberrant production of abnormally glycosylated IgA1 which deposit at renal level in glomeruli.
Subjects included in the study

75 IgAN patients with normal renal function (IgAN-NRF)

VS

75 Healthy blood donor subjects

IgAN-NRF = patients with moderate histological lesions (G1-G2) according to our classification, serum creatinine ≤ 1.2 mg/dl and eGFR >90 ml/min/1.73 m2 body surface area (evaluated by Cockcroft-Gault formula)
miRNA expression profile of PBMCs from IgAN patients and controls

Hierarchical Clustering Analysis of 76 miRNAs

Healthy Blood Donors

IgAN patients

Principal Component Analysis (PCA)
Six miRNAs were identified for their mRNA targets

<table>
<thead>
<tr>
<th>miRNA</th>
<th>mRNA target</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-188-5p</td>
<td>CTNNB1P1, WNT3, GRAP2, B3GALTL,</td>
<td>- Catenin,beta interacting protein 1</td>
</tr>
<tr>
<td></td>
<td>ST6GALNAC1, AKT1S1</td>
<td>- Proto-oncogene protein Wnt-3</td>
</tr>
<tr>
<td>miR-148b</td>
<td>C1GALT1, PTEN, INVS, SOS1</td>
<td>- GRB2-related adapter protein 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Beta-1,3-glucosyltransferase-like</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- GalNAc alpha-2,6-sialyltransferase I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- AKT1 substrate 1</td>
</tr>
<tr>
<td>let-7d</td>
<td>RAS, PTEN, HMGA2</td>
<td>- Core 1 beta1,3-galactosyltransferase 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- phosphatase and tensin homolog</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- inversin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Son of sevenless homolog 1</td>
</tr>
<tr>
<td>let-7b</td>
<td>CCNA, CCND1, CCND3, CDK4, RAS,</td>
<td>- RAS oncogene homolog</td>
</tr>
<tr>
<td></td>
<td>HMGA2</td>
<td>- phosphatase and tensin homolog</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- high mobility group A2</td>
</tr>
<tr>
<td>miR-361-3p</td>
<td>FZD5, WNT7A, NFATC3, PIK3C2B</td>
<td>- Cyclin A, D1, D3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- RAS oncogene homolog</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- high mobility group A2</td>
</tr>
<tr>
<td>miR-886-3p</td>
<td>WNT3, TCF3, JAK3, FOXP4</td>
<td>- frizzled homolog 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Protein Wnt-7a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- nuclear factor of activated T-cells 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- phosphoinositide-3-kinase, class 2, beta polypeptide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Proto-oncogene protein Wnt-3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Transcription factor 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Janus kinase 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- forkhead box P4</td>
</tr>
</tbody>
</table>
miR-148b is a regulator of C1GALT1

C1GALT1 is known as a gene directly involved in IgAN. However, the basis for β1,3-galactosyltransferase reduction in the disease is unknown. Therefore, we evaluated the C1GALT1 mRNA expression levels by real-time PCR (qRT-PCR) in the same set of RNA samples used in the microarray validation.

The inverse correlation observed between the levels of miR-148b and C1GALT1 mRNA supported the bioinformatic analysis showing that this gene is likely a target of miR-148b.
Functional analysis shows that miR-148b modulates C1GALT1 mRNA expression.

**Normal PBMC**
- Transfection of miR-148b mimic
- Measurement of C1GALT1 gene expression (Downregulation)
- Measurement of C1GALT1 protein level

**IgAN PBMC**
- Transfection of miR-148b inhibitor
- Measurement of C1GALT1 gene expression (Upregulation)
- Measure of C1GALT1 protein level

![Graph A](image1)

No = 4
*p < 0.01

![Graph B](image2)

No = 4
*p < 0.01
Data validation of miR-148b

miR-148b expression levels were evaluated by real-time PCR (qRT-PCR) in an independent cohort of 50 IgAN patients and 50 healthy blood donors (HBD). miR-148b levels were found significantly higher in PBMCs of IgAN patients.
Correlation between miR-148b and deglycosylated IgA1

Pearson correlation analysis showed a significant positive correlation, supporting that miR-148b regulates C1GALT1 and the abnormal increase of Gal-deficient IgA1 in IgAN patients is consequent to high expression of miR-148b.

\[
r = 0.4, \ p<0.0001
\]
\[
n = 50
\]
Upregulated expression of miR-148b is specific of IgAN

In order to determine if the up-regulated expression of miR-148b is specific of IgAN, we checked the miR-148b expression in PBMCs from 3 additional disease controls: 3 membranoproliferative glomerulonephritis type I (MPGN-I) patients, 5 focal segmental glomerulosclerosis (FSGS) patients and 10 Henoch–Schönlein purpura (HSP) patients.

We found that the miR-148b were again higher in IgAN patients (p < 0.0001) compared to MPGN-I, FSGS and HSP patients confirming that higher miR-148b levels are typical of IgA nephropathy.
IgA1 DEGLYCOSYLATION PROCESS IN IgAN

- IgA1 DEGLYCOSYLATION PROCESS IN IgAN

O-linked glycans

miR-148b levels

C1GALT1

Hinge Region

Ser/Thr

GalNAcT2

GalNAc

Gal

NeuAc

a2,6sialyl

a2,3sialyl

NeuAc

NeuAc
CONCLUSIONS

• We have identified, for the first time, a miRNA pattern differentially expressed in PBMCs of IgAN patients compared to healthy subjects.

• Some miRNAs are involved in IgAN pathogenesis

• We have biologically demonstrated that miR-148b has a target gene: C1GALT1

• For the first time, it is evidenced an upregulation of miR-148b and downregulation of C1GALT1, which could explain the aberrant glycosylation of IgA1 in IgAN.

• These findings suggest that miR-148b is a marker of IgAN and its inhibition may reverses the lower IgAN typical levels of C1GALT1. Therefore, miR-148b levels may be manipulated to provide useful new therapeutic approaches for the disease.
PRELIMINARY DATA OF miR-148b SERUM LEVELS

Real-time PCR was carried out on 10 IgAN patients and 10 healthy subjects (HS). Our data are normalized on miR-27a (a miRNA highly and equally expressed in all samples).
SOP (Standard Operating Procedure)

1. Take 10 ml of whole blood sample using a tube without any anticoagulant
2. Allow the blood to clot by leaving it at room temperature for 15-30 minutes
3. Centrifuge whole blood at 1800 x g for 10 minutes at 4°C
4. Transfer supernatant (serum) into a clean polypropylene tube and store at -20°C or lower
5. Thaw serum frozen sample and proceed to purification of total RNA, including small RNAs, using the miRNeasy Mini Procedure
6. Reverse transcription PCR to convert isolated RNA into cDNA by miScript Reverse Transcription Procedure
7. Measurement of miR-148b expression by means of miScript Real-Time PCR Procedure
Brevetto N. MI 2010 A002007
Data deposito 28.10.2010
Titolo: Metodo e kit per la diagnosi di 
IgA Nefropatia
Presentata domanda di brevetto 
internazionale PCT

Stakeholders: Biotech Companies
Users: Clinical laboratories
1. Sviluppo del progetto

2. Criticità: preparazione di un business plane

3. Tipo di azione intrapresa di IATRIS: incontri tecnici

4. Tipo di supporto richiesto a IATRIS: incontro con Venture Capitalist