SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TIPOLOGIA DI BORSA RICEVUTA: Borsa di ricerca per soggiorno presso laboratori stranieri

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): metà periodo

TITOLO DELLA RELAZIONE: Role of the long non-coding RNA GAS5 in glucocorticoid response

RELAZIONE:

Background

Glucocorticoids (GCs) are commonly used as therapeutic agents for inflammatory and autoimmune diseases such as inflammatory bowel diseases (IBD), in the treatment of leukaemias and lymphomas. However, considerable inter-individual differences in their efficacy and side effects have been reported and the identification of subjects that are most likely to respond poorly to GCs is extremely important. The molecular mechanisms involved in this variability are scarcely understood and there is presently no means to predict the response in advance.

These agents exert their biological effects by binding to the GC receptor (GR), which translocates from the cytoplasm into the nucleus and binds, through its DNA binding domain (DBD), the glucocorticoid responsive elements (GREs) in the regulatory regions of GC responsive genes. The activated GR can also inhibit directly by binding transcription factors like NF-κB, which control the expression of most proinflammatory genes.

Recent reports showed that the growth arrest-specific 5 (GAS5) gene encodes for a long noncoding RNA (lncRNA) which can act as a riborepressor of the GR. GAS5 interacts with the activated GR, preventing its association with DNA, and consequently suppressing its transcriptional activity. To date, it is unknown if GAS5 has an impact on the GR-dependent repression of NF-κB activity.

In previous studies conducted in our laboratory, peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were treated in vitro with methylprednisolone (MP) and two groups of subjects (good and poor responders) could be identified on the basis of the anti-proliferative effect of the steroid.

In poor responders, higher levels of GAS5 were evident in comparison with good responders, suggesting that this lncRNA could be involved in GC resistance.

Notably, levels of GAS5 in PBMCs were altered also in pediatric patients with IBD after treatment with GCs (paper submitted). No difference was observed in GAS5 levels among steroid sensitive (SS), dependent (SD) and resistant (SR) patients at diagnosis; on the contrary, after four weeks of treatment with prednisone 1 to 2 mg/kg/day, SR patients presented significantly higher levels of GAS5 in comparison with SS and SD groups. In addition, the SD group presented higher levels of GAS5 in comparison with SS patients. Interestingly, patients with unfavourable steroid response (SD + SR) presented higher GAS5 level, further supporting a contribution of GAS5 to steroid ineffectiveness.
A set of experiments were performed to investigate the lncRNA GAS5 as marker involved in GC resistance and its potential role in the GR protein-protein interaction. In particular experiments were conducted: 1) to quantify GAS5 in adult patients with IBD before the treatment with GCs, 2) to quantify GAS5 in pediatric patients affected by acute lymphoblastic leukemia (ALL) at diagnosis, 3) to construct a plasmid expressing GAS5.

Materials and Methods

IBD patients

PBMCs from 21 IBD patients obtained from the biobank of the Laboratory for molecular biomedicine of the Institute of Molecular genetics and Genetic Engineering (IMGGE), collected before starting therapy with methylprednisolone, were used for the molecular analyses. Patients' average age was 39.7 years. Patients were classified on the basis of their clinical response into 3 groups: SR patients who had active disease despite treatment with MP (max 60 mg/day) for 2 weeks, SS patients who did not relapse when therapy was discontinued after tapering and SD patients, who experienced disease relapse within 3 months after the steroid was stopped or during steroid tapering.
This study was conducted after obtaining the approval of The Ethic Committee of Clinical Centre of Serbia, School of Medicine, University of Belgrade, in accordance with the Helsinki Declaration. All participants provided informed consent.

ALL patients

PBMCs from 23 unrelated ALL patients were obtained from the biobank of the Laboratory for molecular biomedicine of the IMGGE. These samples were collected prior therapy with prednisone. Patients' average age was 7.2 years. The marker of sensitivity to GCs was the absolute number of blasts in mm^3 of blood at day 8 since the beginning of GC treatment. If this number was higher or lower than 1000, a patient was considered resistant or sensitive, respectively.
This study was approved by the ethics committee of University Children's Hospital, University of Belgrade. For the molecular analyses, an informed consent was obtained from the parents or guardians of each ALL patient.

Cell line

The HeLa human cervical carcinoma (ATCC, CCL-2) cell line was grown in DMEM medium (Sigma-Aldrich, USA) supplemented with 10% FCS, 1% L-glutamine 200 mM, 1% penicillin 10000 UI/mL and streptomycin 10 mg/mL. Cell culture was maintained according to standard procedures in a humidified incubator at 37 °C and with 5% CO_2.

Expression studies

Total RNA was extracted from cells in TRI reagent solution (Ambion, USA), following the manufacturer's instructions. The concentration and purity of RNA were measured spectrophotometrically at 260 and 280 nm. cDNA was synthesized from RNA using the RevertAid Reverse Transcriptase (Thermo Scientific, USA), following the manufacturer's instructions. GAS5 expression levels were quantified by real-time PCR (Applied Biosystems 7900, CA, USA) using the TaqMan gene expression assay kit (Thermo Scientific, USA). In order to normalize the obtained values, GADPH gene expression was used as an internal control. The expression levels of GAS5 were calculated using the ΔΔCT method.

Plasmid

PCR product obtained from cDNA Hela cells using GAS5_F_TTTCGAGGTAGGAGTCGACT and GAS5-R_GGAGACACTGTTTTAATCT primers was cloned into pGEM-Teasy plasmid. pGEM-Teasy-GAS5 vector was then sequenced in both directions with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on 3130 Genetic Analyzer (Applied Biosystems, USA) using universal M13 forward and reverse primers to verify the sequence. Next, the insert was extracted using restriction enzymes and then was subcloned into pcDNA3.1 plasmid. After that, the insert was sequenced again as reported above. The pcDNA3.1_GAS5 plasmid was amplified in DH5α *E. Coli* strain and then extracted using ZymoPURE Maxi Prep Kit (Zymo Research, USA).

Transfection

For GAS5 plasmid transfection, Hela cells were seeded in 12-well plates at $2x10^5$ per well. 24 h later, cells were transfected with the empty vector or the pcDNA3.1_GAS5 plasmid using Lipofectamine®2000 Transfection Reagent (Invitrogen, USA) according to the provided protocol. LncRNA GAS5 plasmid amount was 1 or 2 µg per well. The medium was replaced 5 h after the transfection and GAS5 expression was evaluated after 24 h. To evaluate the transfection efficacy, the cells were transfected with pCH110 plasmid, which contains the E. Coli *lacZ* reporter gene, encoding β-galactosidase.

Statistical analysis

Statistical analyses were performed using Graph-Pad Prism version 4.00 (GraphPad, La Jolla, CA, USA). Mann Whitney test was used for the analysis of gene expression. P values < 0.05 were considered statistically significant.

Results and discussion

1) GAS5 in IBD patients

Among 21 patients enrolled, 2 were SR, 9 SD and 10 SS. Considering the small number of SR patients, SD and SR patients were analysed together as an unfavourable steroid response group (SD + SR).
As reported in the figure 1, no difference was observed in GAS5 levels between the two groups of patients, confirming data reported in literature on healthy donors and on pediatriac IBD patients (data submitted).

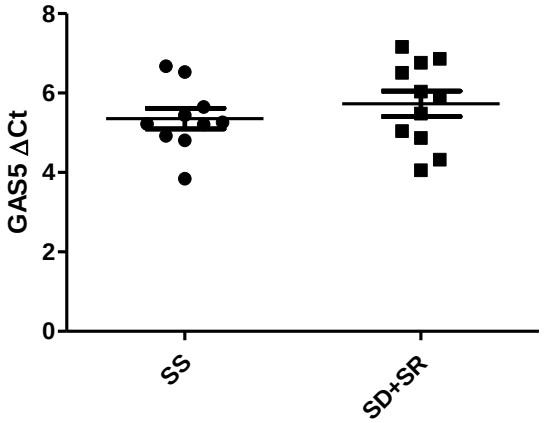


Fig. 1 GAS5 expression in SS and SD+SR patients before the treatment with methylprednisolone. Mann Whitney test p-value= 0.37.

Considering that the levels of GAS5 seem to be predictive of clinical response only after treatment with GCs, an in vitro treatment with GC for 72 h will be performed on PBMCs obtained from a new group of IBD patients.

2) GAS5 in ALL patients

Among 23 patients enrolled, 6 were SR and 17 SS. As reported in the figure 2, no difference was observed in GAS5 levels between the two groups of patients, demonstrating that also in other diseases, like ALL, GAS5 levels are not predictive of GC response.

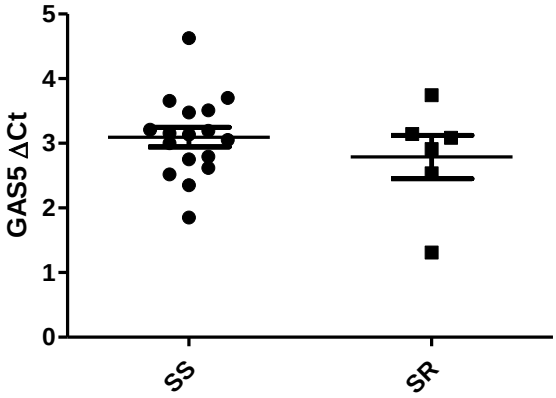


Fig. 2 GAS5 expression in SS and SR patients before the treatment with GC. Mann Whitney test p-value=0.55.

The next aim will be to measure GAS5 in the same patients after 15 and 30 days of treatment and to correlate its levels with the clinical response.

Plasmid construction and efficacy of transfection

The pcDNA3.1_GAS5 was run on agarose gel alongside the empty pcDNA3.1 to verify the different size between the two vectors (Fig. 3). Next, the sequencing analyses confirmed the presence of the correct insert into the plasmid pcDNA3.1.

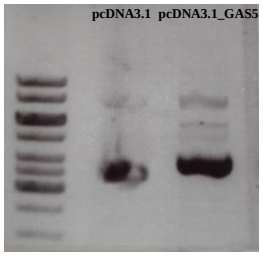


Fig 3. Empty pcDNA3.1 and pcDNA3.1_GAS5 in agarose gel showed a different electrophoretic profile indicating the presence of the insert in pcDNA3.1_GAS5.

In order to optimize the transfection protocol the cells were transfected with 1 or 2 µg of pcDNA3.1_GAS5 and empty pcDNA3.1. In parallel the cells were also transfected with 1 or 2 µg of pCH110 plasmid. As shown in figure 4, the levels of GAS5 increased after transfection with pcDNA3.1_GAS5: in particular, the quantity of GAS5 was higher using 2 µg of pcDNA3.1_GAS5, as expected. Similar results were obtained when the cells were transfected with 1 or 2 µg of pCH110 plasmid (Fig. 5) demonstrating high efficiency transfection of cells.

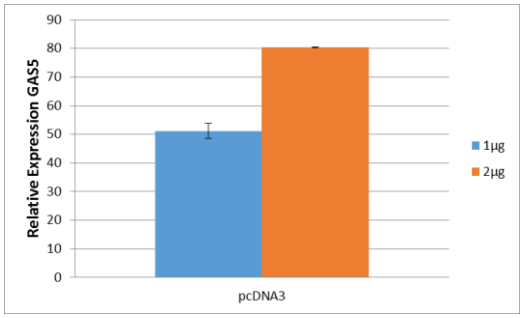
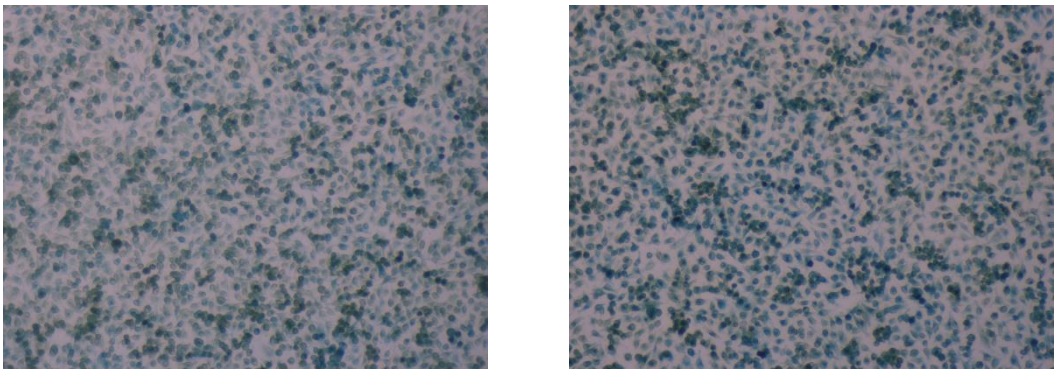


Fig 4. GAS5 relative expression after transfection with two different concentrations of pcDNA3.1_GAS5 respect to empty pcDNA3.1.



1µg 2µg

Fig 5. Transfected cells expressing the lacZ gene: β-galactosidase catalyses the hydrolysis of X-Gal producing a blue precipitate that can be visualized under a microscope. The percentage of cells in blue color increased using 2 µg of pCH110 plasmid.

The next step will be to treat HeLa cells overexpressing GAS5 with GC and then to perform EMSA (Electrophoretic Mobility Shift Assay), in order to evaluate if the lncRNA has an impact on the GR-dependent repression of NF-κB activity.