SOCIETÀ ITALIANA DI FARMACOLOGIA

RELAZIONE DI FINE PERIODO

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TIPOLOGIA DI BORSA RICEVUTA: SIF-MSD Italia

TIPOLOGIA DI RELAZIONE: Relazione finale

TITOLO DELLA RELAZIONE: Identification of novel biomarkers for assessing sensitivity to steroid treatment in pediatric idiopathic nephrotic syndrome

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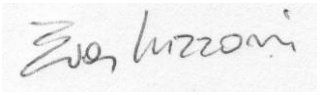

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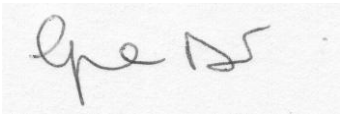

SOCIETÀ ITALIANA DI FARMACOLOGIA

RELAZIONE:

Background

Idiopathic nephrotic syndrome represents the most common type of primary glomerular disease in children and affects 16–17 per 100,000 children. The onset of the disease occurs usually between the ages of 2 and 8 years, with a peak of incidence between 3 and 5 years [Eddy AA and Symons JM, 2003; Gipson DS et al., 2009]. The physiopathologic mechanisms of INS have not been completely clarified yet; however, the disease is triggered by an increase in glomerular permeability caused by an abnormal immunologic response, that results in an alteration of the capillary structure and of the integrity of the glomerular membrane [Eddy AA and Symons JM, 2003].

Glucocorticoids (GCs) are the first-line therapy, even if considerable interindividual differences in their efficacy and side effects have been reported. Indeed, although steroids are able to induce remission in 90% of cases after four-six weeks of treatment, within those patients almost 50% will present an optimal response to steroid treatment, with no relapse or infrequent relapses (NR-IR), while almost 40% show frequent relapses and become steroid dependent (FR-SD). Moreover, 10% of patients will never respond and are therefore steroid-resistant (SR) [Pasini A et al.,2015; Lane JC and Kaskel FJ, 2009].

Immunosuppressive and anti-inflammatory effects of GCs are mainly due to the transcription regulation of pro- and anti-inflammatory genes. This mechanism of action is the result of a complex multistep pathway that involves the GC receptor and several other proteins, encoded by polymorphic genes.

Steroid responsiveness, in INS, is of major prognostic importance as resistant patients are prone to progressive disease and renal failure and dependent patients are at risk of more aggressive treatment and of drug- and disease-related complications.

To date, a number of demographic and/or clinical markers have been examined in correlation with GC response in INS, but results have not been consistently replicated. Genetic and epigenetic markers are likely to complement clinical and demographic predictors: phenotypes resulting from genetic changes, such as single nucleotide polymorphisms, and epigenetic modifications, such as DNA methylation, can markedly influence drug mechanism of action and alter efficacy profiles.

The main objective of this project is the identification of molecular determinants studying genetic and epigenetic mechanisms that lead to clinical response variability to glucocorticoids. The markers will allow to distinguish the different subgroups of patients (resistant, dependent and responsive) affected by INS in order to personalize the glucocorticoid treatment.

Materials and methods

Samples, DNA extraction and quantification

Patients with INS at onset were enrolled for a prospective multicenter Italian trial on the treatment of INS (ClinicalTrials.gov ID: NCT01386957) with the aim of studying the benefits and potential adverse effects of a prolonged initial corticosteroid regimen. Children with a first episode of INS, presenting at 49 Pediatric and Pediatric Nephrology Units in 10 Italian regions, were treated with prednisone at a dose of 60 mg/m2/day for either 4 or 6 weeks, depending on whether time to remission was < or > 10 days. Steroids were then tapered over a 16-week period. Total prednisone dosage was 2,828 mg/ m2 in subjects achieving remission within 10 days, 3,668 mg/m2 in the others. Patients were classified into three groups: steroid-sensitive (SS: no relapse/infrequent relapse subjects), steroid-dependent (SD: frequent relapse/steroid-dependent subjects) and steroid-resistant (SR), as defined by Cuzzoni et al. [2016]. Ethics Committee approval was

obtained from all the participating centers. The parents of all the participating children gave written informed consent before the study began.

Total genomic DNA was isolated from peripheral blood using a commercial kit (Gene Elute Blood Genomic DNA kit, Sigma Aldrich, Milan, Italy). For all patients, DNA was quantified on the Qubit 2.0 fluorometer (Thermo Fisher) using the Qubit dsDNA HS Assay Kit (Thermo Fisher).

Library construction

Primers for the library were designed with Ion Ampliseq Designer (https://www.ampliseq.com) from Life Technologies. A panel was designed including 216 genes coding for proteins involved in the mechanism of action of different drugs: GCs, biologics, antimetabolites. The Ion Ampliseq Designer, due to the large amounts of genes in the panel, generates 2 different pools of primers reducing therefore the possibility of cross-reaction between primers. Library construction was performed using the Ion AmpliSeq™ Library Kit 2.0 (Thermo Fisher) according to the manufacturer's guide, including four major steps: amplification, digestion, ligation and purification, and quantification. First, genomic DNA targets were amplified: 10 ng of input DNA were processed on the GeneAmp® 9700 System (Thermo Fisher) using the thermal cycling conditions of enzyme activation for 2 min at 99 °C, amplification for 15 cycles of 15 s at 99 °C followed by 4 min at 60 °C, and hold at 10 °C. This amplification reaction was performed for every patient with both pools of primer, the two amplified pools sequences were then mixed together for the further steps. Second, primer sequences were partially digested using the enzyme FuPa: a total of 2 µl FuPa reagent was directly added into each PCR product and then incubated for 10 min at 50 °C, 10 min at 55 °C, 20 min at 60 °C and hold up to an hour at 10 °C. Third, barcode adapters were ligated to the amplicons and purified: a total of 4 µl Switch solution, 2 µl diluted Ion Xpress™ barcode and P1 adapter mix and 2 µl DNA ligase were consecutively added into each of 22 µl digested amplicon and followed by incubation for 30 min at 22 °C, 10 min at 72 °C and hold up to an hour at 10 °C. Each unamplified library was purified using 1.5 × Agencourt® AMPure® XP reagent (Beckman Coulter, FL, USA) according to the manufacturer's guides. Fourth, unamplified libraries were quantified, using 7900HT Fast Real time PCR System (Applied Biosystem) with the KAPA Library Quantification Kit (Resnova) and then diluted to 10 pM.

Template preparation and NGS sequencing

A total of 10 µl pooled libraries mixed with 15 µl nuclease-free water were subjected to emulsion PCR (emPCR) on the Ion OneTouch™ 2 instrument (Thermo Fisher) with the Ion PGM™ Template OT2 200 Kit (Thermo Fisher). Template-positive Ion Sphere Particles (ISPs) were enriched on the Ion OneTouch™ ES instrument (Thermo Fisher) according to the manufacturer's protocol.

NGS was performed on the Ion Torrent PGM™ platform (Thermo Fisher) using the Ion PGM™ Sequencing 200 v2 Kit following the manufacturer's instruction. Four barcoded libraries were pooled together and loaded onto each Ion 318™ Chip v2.

Data processing

Raw data were processed by the Ion Torrent browser (Thermo Fisher) using Homo sapiens hg 38 as reference sequence. The Coverage Analysis plugin was employed to provide statistics and graphs describing the level of sequence coverage produced for targeted regions and to generate a Barcode Summary Report, detailed with mapped reads (total number of reads mapped to the reference), on target percentage (the percentage of reads mapped to any targeted region relative to all reads mapped to the reference) , mean depth (the average number of times any single target base was read) and uniformity percentage (the percentage of bases in all targeted regions covered by at least 0.2x the mean depth) for each sample in the

same run. Caller Variant Analysis was applied, which allows to detect variations in the nucleotide sequence. This analysis generates a VCF files (Variant Caller Format). The software wANNOVAR was used to generate the VCF annotated files with specific information relative to the variant (chromosomal location, type of mutation, rs number, coverage, damaging variant). Data were then analyzed using pgADMIN version 4.

Methylation analysis of CASP1 and NRLP3

Genomic DNA samples were analyzed at CpG sites in the CASP1 (cg13802966) and NLRP3 (cg21991396) gene promoters to assess DNA methylation levels. Following bisulfite treatment of the DNA samples, the extent of methylation was measured through the use of a fluorescent single nucleotide primer extension (SNuPE) assay which was then resolved using denaturing polyacrylamide gel electrophoresis. Gels were imaged using a LiCor Odyssey Fc Imaging System and signals from the labeled primers were quantified with Image Studio Software. Comparing the incorporation of Cy3-dCTP versus Cy3-dUTP in the bisulfite-treated DNA at each of these two loci using SNuPE, we were able to determine the relative levels of 5-methylcytosine for each patient at these sites. Using the SNuPE assay, we were able to estimate methylation using the signal intensities from Cy3-dCTP and Cy3-dUTP incorporation. The level of methylation was quantified with the equation: Cy3-dCTP signal/(Cy3-dCTP signal + Cy3-dTTP signal.

Statistical analysis

For continuous variables, normality of distribution was assessed by means of visual examination of the data plot and a Shapiro test. Logarithmic transformation was applied to normalize distribution and/or reduce variance. The correlation between continuous variables was assessed using the appropriate parametric (Pearson) and nonparametric (Spearman) tests. Any possible association between DNA methylation status and clinical variables (response, age at the onset of disease, and sex) was investigated using univariate logistic regression models. Receiver operating characteristic (ROC) curves were constructed for the DNA methylation status to determine the optimal cutoff value for discriminating between patients' clinical response to steroid treatment. Sensitivity, specificity, and the positive and negative predictive values (PPV, NPV, respectively) of the cutoff point were analyzed. Logistic regression, considering the proportion of patients achieving the predicted clinical response, comparing patients who reached the optimal cutoff point and those who did not, was used to confirm the significance of the cutoff values. Statistical analyses were performed using the software R. P values lower than 0.05 were considered statistically significant. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated for all the analyses.

Results for next generation sequencing analysis

Eighty-eight patients (median age: 4.2 years) were enrolled from a previous pharmacodynamic study (Cuzzoni et al., 2016) for the NGS project. Demographical and steroid response characteristics of these patients are reported in table I. A statistically significant effect of age was observed between SS, SD and SR patients (multinomial logistic regression, p-value=0.028); in particular there was a significant difference between SD and SR patients (p-value=0.016, OR=1.29, 95%CI=0.86-0.57) and a trend between SD and SS patients (p-value=0.06, OR=1.18, 95%CI=1.41-0.99). Sex distribution was not different among the three groups of patients.

All patients	**SS (n = 45)**	**SD (n =29)**	**SR (n =14)**
Male, n (%)	32 (71%)	19 (66%)	8 (57%)
Median age (range)	4.8 (2-15)	2.9 (1-14)	6.7 (0-17)

Table I: demographical characteristics of NS patients divided in the three groups on the basis of clinical response

The DNA of fourty-four patients has been tested yet, coverage statistics was comparable among all runs and was in the range of accepted quality criteria. The files have been processed by wANNOVAR and contain all the information over the annotated sequence of our patients. The panel sequenced 216 genes involved in different drugs pathways. Among these, 46 are involved in steroid mechanism of action (pathway, drug target, pathway transcription regulation). To allow interpretation of the data, we transferred the files into a relational database developed with PostgreSQL (open-source relational database technology). This allowed us to screen the large amount of data obtained and to identify information of interest in this study.

From the analysis performed we could observed an unknown variant that was present only in a patient clinically resistant to steroid therapy. This variant was a deletion of an A in the coding region of the gene Trinucleotide repeat-containing 6A (*TNRC6A)* in the chromosome 16, position 24795919. This deletion leads to a frameshift with a consequent non active protein. The patients presents this variation in homozygosis and none of the other patients showed the same variant neither in homozygosis nor in heterozygosis.

Two other variants in homozygosis have been observed analyzing this cohort of idiopathic nephrotic syndrome patients, both present in steroid-dependent patients. The first one was an unknown deletion of a GC fragment on the sequence coding for the gene *TNRC6B* on chromosome 22, position 40323121, leading also in this case to a frameshift in the protein sequence. The second one was a known variant on the *PYGL* gene (rs11356035) that has been shows to have an impact on the splicing of the relative protein.

Moreover, different variants in heterozygosis have been found both in steroid-resistant patients than in patients showing clinical dependency, all with a high impact on the relative protein. In particular, for the resistant patients, 7 variants have been observed on genes related with the mechanism of glucocorticoids action: *GSTT1*, *CRISPLD2*, *CNOT1*, *DROSHA*, *TBP*, *RELA* and *TPMT.* While for de dependent patients, 20 variants have been observed in different genes: *TNRC6A, TNRC6B, TBP, NOS3, TPMT, CNOT1, CRISPLD2, CASP1, DROSHA, XPO5, ABCB1, GATA3, PYGL, SERPINA6, CREBBP, CD3EAO, ERCC1, NCOA3* and *ST13*.

Results for methylation analysis

In collaboration with the laboratory of Prof. W. Evans at St. Jude Hospital of Memphis, we selected 42 patients from our INS pediatric cohort (the first 14 patients for each group of clinical response). DNA samples of these patients have been analyzed in order to assess the DNA methylation of CASP1 and NLRP3 and the possible correlation of methylation and clinical response was evaluated.

For CASP1 the DNA methylation in our INS patients was not detected, while for NLRP3 median methylation value was 0.44 (inter quartile range 0.16-0.51). Normality of distribution was assessed by means of visual examination of the data plot and a Shapiro test.

A statistical correlation with age at the onset of the disease was evident for NRLP3 methylation (Pearson's test p-value = 0.002; figure 1 left); while no differences was observed with sex. (Figure 1 right)

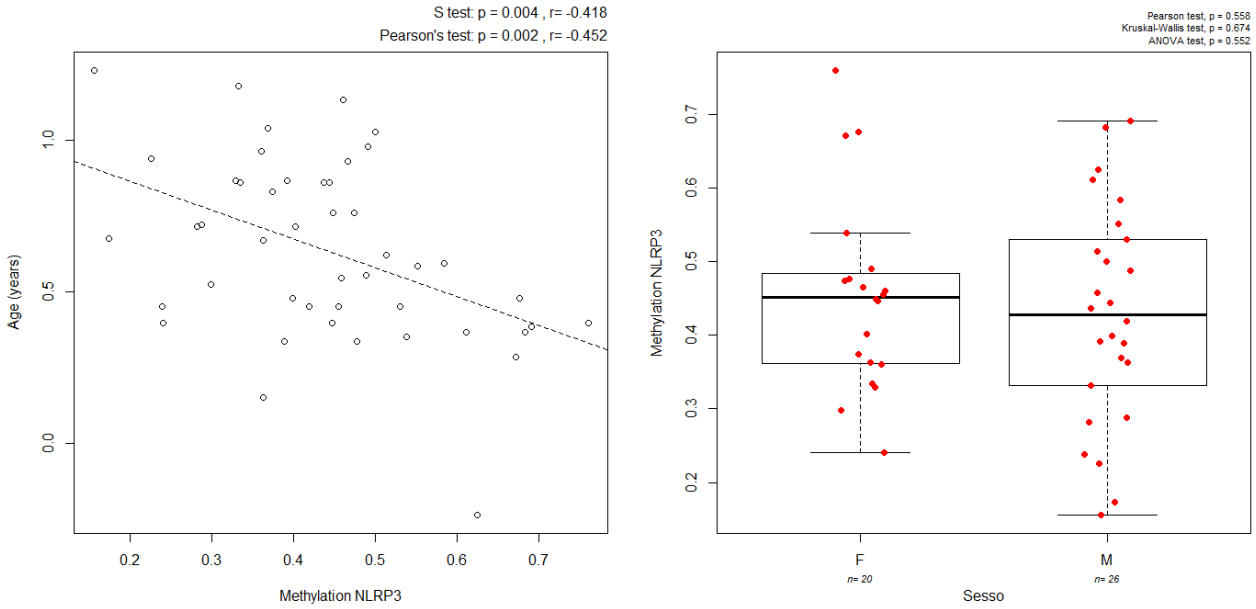

Figure 1: left) Scatterplot displaying age at the onset of the disease and NLRP3 methylation; right) Boxplot comparing sex and DNA methylation of NLRP3. NRLP3 methylation is plotted in Log10 scale. The bold horizontal line represents the distribution mean.

Considering DNA methylation of NLRP3 promoter and clinical response, no statistical significance has been observed to distinguish sensitive and resistant patients, probably due to the small group of patients. Therefore, in order to analyze a higher number of patients, in collaboration with the group of dott. Gianluigi Zaza, at the University of Verona, we have analyzed a cohort of adults patients with nephrotic syndrome, treated with steroids and classified on the basis of their clinical response: 14 steroid resistant and 18 steroid sensitive patients. Even in this cohort of patients CASP1 was not methylated, while different NRLP3 methylation levels were observed (median 0.31, IQR 0.21-0.35). No statistical differences have been observed in this cohort in terms of age and sex.

Considering the two cohorts of patients, NRLP3 methylation levels were able to significantly distinguish between steroid resistant (SR) and steroid sensitive (SS) patients (logistic regression analysis p-value = 0.017; figure 2).

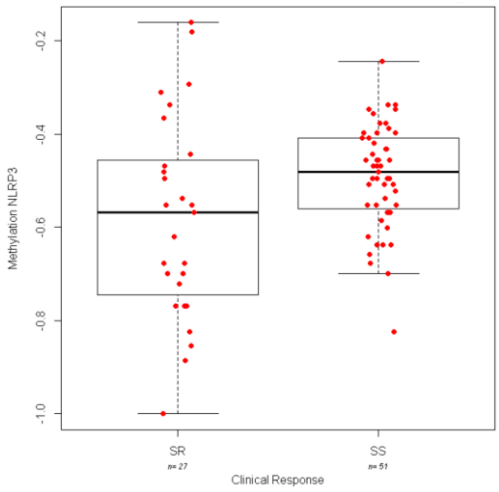

Figure 2: Boxplot comparing DNA methylation of NLRP3 and clinical response. NRLP3 methylation is plotted in Log10 scale. The bold horizontal line represents the distribution mean.

Receiver operating characteristic (ROC) curves was constructed to assign optimal cutoff values for NLRP3 methylation: an optimal cutoff of 0.215 could be defined, and was able to distinguish SR patients from the others. The area under the ROC curves (AUC) was 65.0 % (Figure 3). The test had a sensitivity of 94.1% and a specificity of 44.4% (positive predictive value [PPV] 80.0%; negative predictive value [NPV] 76.2%). Logistic regression analysis confirmed a higher proportion of SR patients among those who had lower NLRP3 methylation then the cutoff point (p-value $5x10^{-5}$, OR 0.07, 95% CI 0.02–0.28) in comparison with those who present higher methylation levels.

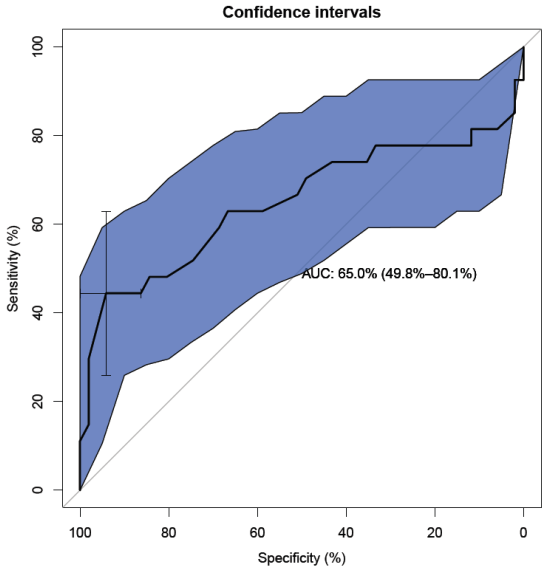

Figure 3: Areas under the ROC curves of NRLP3 methylation among 27 SR and 51 SS nephrotic syndrome patients

CONCLUSION

Nephrotic syndrome is a rare disease characterized by edema, massive proteinuria and hypoalbuminemia. The current mainstream therapy consists of glucocorticoids, which induce remission in 90–95% of the patients. Steroid responsiveness is of major prognostic importance: patients with steroid dependence and resistance are at risk of more aggressive treatment and disease-related complications. Many efforts have been made to predict steroid response in children with the disease; however, to date, no definitive prognostic factor has been defined.

In this project, we focused our attention on genetic and epigenetic variations that could influence glucocorticoid response in order to find new biomarkers able to predict the clinical response to glucocorticoids before starting treatment.

Performing sequencing analyses we found that a variation in the sequence coding for the protein TNRC6A could be related of glucocorticoid resistance. The TNRC6B gene mediates the miRNA-guided mRNA cleavage. MiRNAs regulate gene expression at the post-transcriptional level by binding to the 3´ untranslated region (UTR) of a target gene. This can lead to inhibition of translation or enhanced degradation of their target mRNAs. Through this mechanism, miRNAs can regulate a large number of genes. Consequently, variations in miRNA function could affect many targets with functional consequences. The missense A deletion in the gene coding for TNRC6A SNP was predicted to abort protein function, this change could perturb the maturation of the mRNA, changing the sequence of the protein, and therefore contribute to the alteration of the mechanism of action of glucocorticoids, indeed it has been show that the depletion of TNRC6 leads to the upregulation of many mRNA targets [Eulalio A, 2009].

Moreover, in this study we investigated the methylation status of CASP1 and NLRP3 genes finding, for the last gene, an under-methylation status in steroid-resistant patients. We identified an optimal cutoff of 0.215 with a high sensitivity and a good specificity, that was able to significantly identify resistant patients. Our findings are at least partially in accordance with Paugh et al (2015) that, in a recent study, revealed a novel mechanism by which caspase 1 (CASP1) and its activator NLRP3 (NLR family, pyrin domain containing 3) modulate the biological and pharmacological effects of glucocorticoids via cleavage of the glucocorticoid receptor. In particular, they found that leukemia cells exhibiting higher expression of CASP1 and NLRP3 had significantly lower methylation of their promoter regions compared to glucocorticoid sensitive acute lymphoblastic leukemia cells, and suggested that this mechanism could also modify glucocorticoid effects in other diseases.

If confirmed in larger cohorts, these two genetic and epigenetic markers related to glucocorticoid response, could be used in the attempt to reduce treatment failures and to improve the quality of life of the patients, leading therefore to a superior level of therapy for nephrotic syndrome patients. In addition these biomarkers could be evaluated even in other pathologies, such as rheumatoid arthritis and chronic inflammatory bowel disease.

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