ABERRANT METHYLATED DNA REGIONS LEAD TO LOW ACTIVATION OF CD4+ T CELLS WITH A CONSEQUENT IMBALANCE OF THE TH1/TH2 POLARIZATION IN IGA NEPHROPATHY PATIENTS

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INTRODUCTION AND OBJECTIVES

Immunoglobulin A nephropathy (IgAN) is the most common form of primary glomerulonephritis worldwide among patients undergoing renal biopsy. The pathogenesis of this disease seems to have a strong genetic component but so far, no genetic variants or genes underlying these loci have been identified as causative or affecting the pathology (1). In this setting, also the DNA methylation could be an important factor influencing the pathology (2). Rapid advances in the field of epigenetics are now revealing a molecular basis for how heritable information other than DNA sequence can influence gene function (2-4).

To assess possible changes in CpG methylation in IgA nephropathy, we analyzed the CpG methylation in a genome-wide manner on the DNA derived from CD4+ cells of 6 IgAN samples and 6 healthy controls.

METHODS

DNA methylation analysis was performed by the Illumina Human-Methylation450 BeadChip that interrogates DNA methylation at more than 485000 CpGs. All statistical analysis was performed using R and the RnBeads R package for comprehensive analysis of DNA methylation data. Identified CpG, differentially methylated, were further validated on ten different samples of IgAN and HS. Gene expression studies by Real-time PCR were performed on identified methylated genes/promoters to verify the correspondence between the methylation status and the gene expression.

	IgAN	HS
Number	16	16
Male/Female	10/6	13/3
Age (years)	49.1 ± 11.3	43.9 ± 9.2
sCr (mg/dL)	1.2 ± 0.4	n.d
eGFR(mL/min/1.73m^2)	82.0 ± 40.0	n.d
Proteinuria (g/24h)	0.9 ± 1.1	n.d
Systolic BP (mmHg)	131.3 ± 15	118 ± 13
Diastolic BP (mmHg)	79.7 ± 7	71 ± 5

Abbreviations: eGFR, estimated glomerular filtration rate calculated with the Cockcroft–Gault formula; sCr, serum creatinine. Values are expressed as mean±s.d.

Table 1: Demographic and clinical features of patients and healthy participants

RESULTS

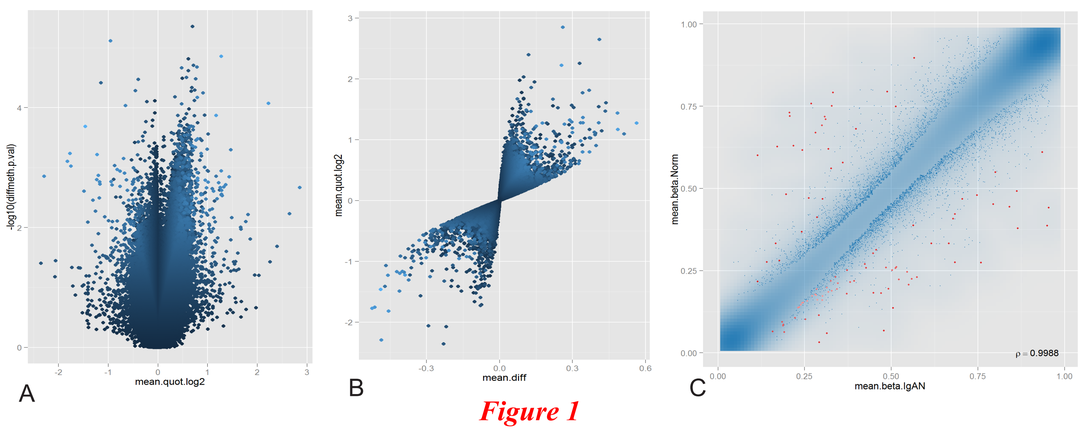

Figure 1

Figure 1. (A) Differential methylation analysis between 6 IgAN subjects and 6 HS was conducted both on site and region level according the combinedRank parameter. We selected the best combined ranks having a threshold of a ($\Delta\beta > 0.15$ and a $p < 0.05$. (B) We found 281 CpG sites differentially methylated in IgAN patients respect to HS, 138 hypermethylated and 143 hypomethylated.

(C) Differential methylation on the region level conducted with the same threshold criteria showed some specific areas differentially methylated in IgAN patients respect to HS . We found 60 tiling regions (windows of 5kb) (24 hypermethylated and 36 hypomethylated), 5 gene bodies (4 hypermethylated and 1 hypomethylated), and 4 CpG islands (2 hypermethylated and 2 hypomethylated).

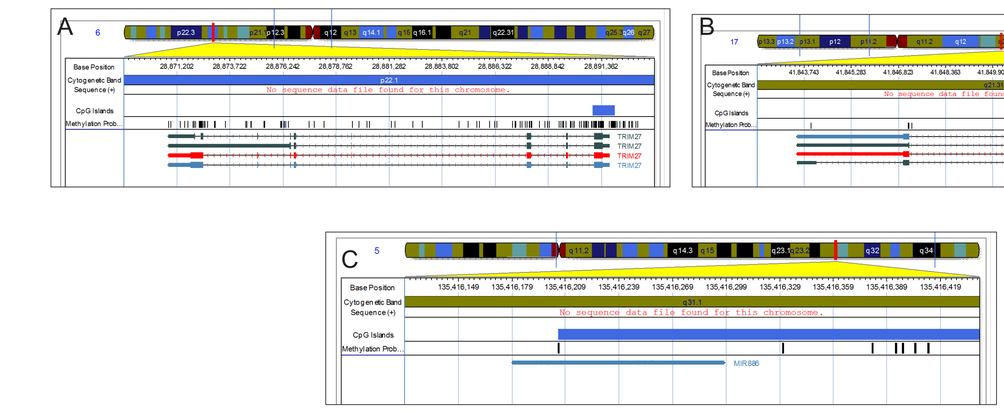

Figure 2

Figure 2. Some most significant differentially methylated regions included genes specifically expressed in T cells and involved in the T cell receptor signaling. In particular, we found TRIM27 (A) and DUSP3 (B) hypomethylated in correspondence to the promoter region and the 3'UTR region, respectively. They function as signal transductors of the T cell receptor. Moreover, in the chromosome 5 we found the VTRNA2-1 gene (known as precursor microRNA miR-886) (C) as one of most strongly and extensively hypermethylated region in IgAN patients respect to HS. It is methylated in the promoter at the 200 bp block upstream of the transcription start site and it is also part of a CpG island. Blue box in the figures represent CpG islands. Black vertical lines represent methylation probes.

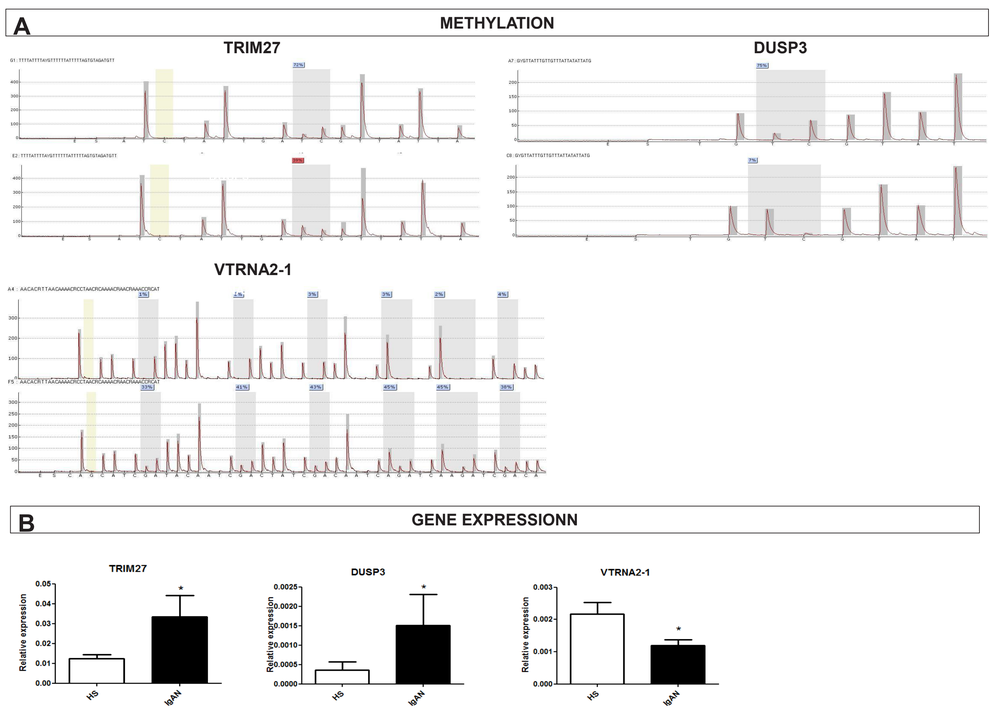

Figure 3

Figure 3 (A): Representative pyrogram of methylation in 10 IgAN patients and 10 HS for TRIM27, DUSP3 and VTRNA2-1. Methylation in TRIM27, DUSP3 and VTRNA2-1 was validated by pyrosequencing on the same regions found methylated in the whole genome assay on 10 IgAN patients and 10 HS. We confirmed that TRIM27 and DUSP3 were hypomethylated in IgAN patients ($p<0.05$). The VTRNA2-1 region was confirmed hypermethylated in IgAN patients with a mean difference in methylation levels of 25%. (B): We studied the gene expression of these three genes in the CD4+ T cells of the same 10 patients and 10 HS. The genes were differentially regulated in IgAN patients respect to HS and were regulated inversely to the methylation of the corresponding DNA region.

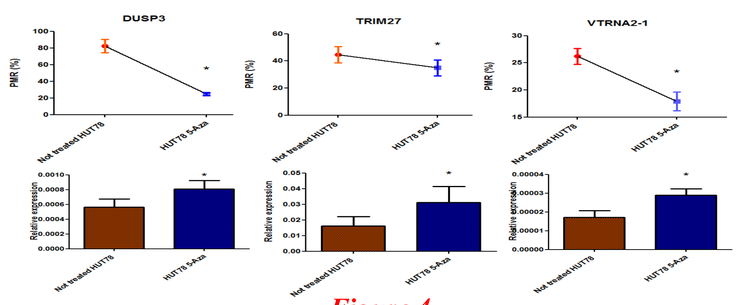

Figure 4

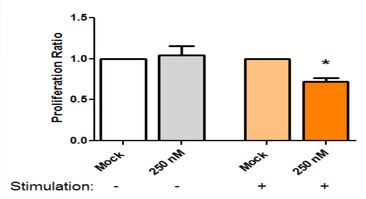

Figure 5

Figure 4. To further confirm the regulatory role of DNA methylation in DUSP3, TRIM27 and VTRNA2-1 expression, we treated the CD4+ HUT-78 cells for 2 days with the DNA methyltransferase inhibitor 5-Aza-20-deoxycytidine which causes DNA demethylation or hemi-demethylation. DNA demethylation can regulate gene expression by "opening" the chromatin structure. We then determined both the DNA methylation status of these three DNA region and the gene expression by methylation specific primer (MSP) analysis and Real-time PCR, respectively. Methylation levels within these regions decreased from 92% in the mock-treated cells (Mock) to 21.5% for DUSP3, from 37.9% to 28.2% for TRIM27 and from 26.2 to 17.2% for VTRNA2-1. Conversely, the gene expression levels increased of 1.5-fold for DUSP3, 1.9-fold for TRIM27, and 1.7-fold for VTRNA2-1, respectively. **Figure 5.** Since the miR-886 codified by VTRNA2-1 was found responsible for the regulation of the cell proliferation (5), we studied whether also in CD4+ T cells it can regulate the proliferation rate. Results showed that, the transient transfection of CD4+ T cells with 250 nM of miR-886 precursor inhibitor led to a decrease of the proliferation rate of 30% ($p<0.02$). The proliferation reduced only when cells were stimulated with CD3 and CD28 molecules. Instead, no difference was detected in unstimulated cells.

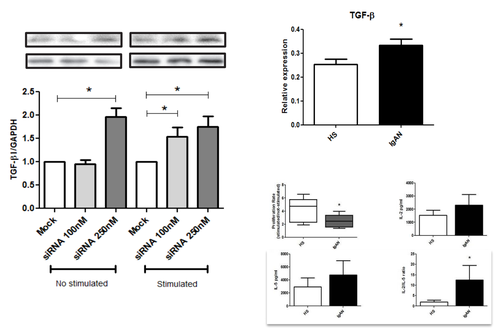

Figure 6

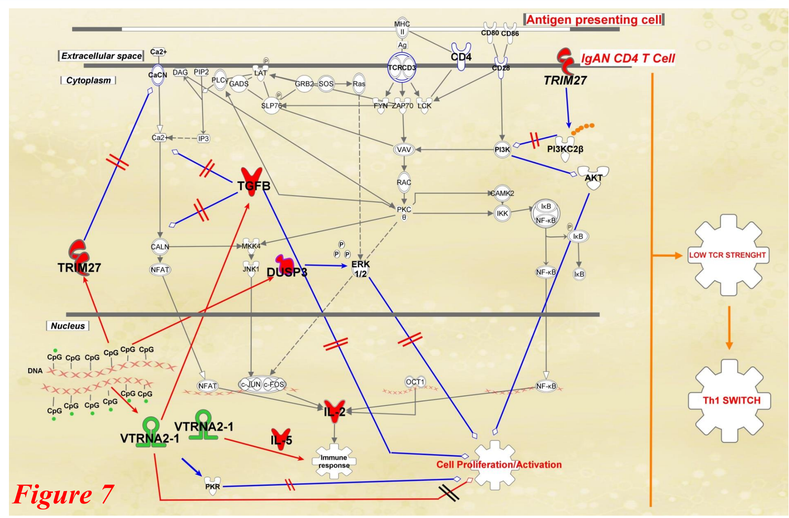

Figure 7

Figure 6.: The non-coding RNA codified by VTRNA2-1 regulates the TGF-β expression in CD4+ T cells. Notably, also TGF-β, similarly to TRIM27 and DUSP3, can hamper the early steps of T-cell activation by reducing the calcium flux or by attenuating signaling downstream of the TCR. C: CD4+ T cells of IgAN patients have an impaired cell proliferation and an elevated IL-2/IL-5 ratio. IL-2/IL-5 ratio was significantly higher in IgAN patients indicating a shift towards Th1-like cells.

Figure 7. DUSP3 dephosphorylates and inactivates ERK1 and ERK2 that are involved in cell growth, proliferation and survival. TRIM27 functions as a RING E3 ubiquitin ligase that decreases PI3K enzyme activity through the polyubiquitination of PI3KC2β. It also negatively regulates CD4+ T-cells by inhibiting KCa3.1 channel activity and TCR-stimulated Ca2+ flux. At the same time, the DNA region encoding the VTRNA2-1 nc-RNA was found to be hypermethylated leading to its down-regulation. In turn, following CD3/CD28 TCR stimulation, the lower levels of VTRNA2-1 cause a decrease in the CD4+ T-cell proliferation, plausibly through the activation of the interferon-inducible kinase PKR. The lower activation of CD4+ T-cells and the lower TCR strength can determine, in some biological settings, Th1 polarization with higher IL-2 production.

CONCLUSIONS

We described, for the first time, some specific chromosomal regions abnormally methylated in IgAN patients, some of which including genes involved in the T cell receptor (TCR) signalling and in the CD4+ T cell response and proliferation. These methylated regions led to the altered expression of genes of the TCR signal transduction, indicating an atypical response of the CD4+ T cells of IgAN patients.

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