Expression Profile of Long Non-Coding RNAs (lncRNAs) in Serum of Patients with Multiple Sclerosis

Massimo Santoro1, Matteo Lucchini 2, Chiara De Fino2, Francesco Antonio Losavio2, Massimiliano Mirabella2, Viviana Nociti1,2

1 Don Gnocchi Foundation, Milan, Italy
2 Policlinico "Gemelli "Foundation, Catholic University of Sacred Heart, Rome, Italy

INTRODUCTION

The pathobiology of Multiple Sclerosis (MS) includes inflammatory and neurodegenerative mechanisms that affect both white and gray matter. These mechanisms underlie the relapsing, and often eventually progressive, course of MS, which is heterogeneous; confident prediction of long-term individual prognosis is not yet possible. Thus, guiding research towards distinguishing reliable biomarkers for every independent MS pathogenic factor is of primary importance. There is now a growing interest in long non-coding RNA (lncRNAs) as potential biomarkers that could provide information predicting disease activity and progression. LncRNAs are a heterogeneously group of non-coding transcripts (200 nucleotides in length) that can inhibit or promote transcription through histone and chromatin alteration, alter splicing profiles, or mask miRNA binding sites. In a preliminary study we showed specific over-expression of three circulating lncRNAs in the serum of relapsing-remitting MS (RR-MS) patients: *nuclear paraspeckle assembly transcript 1* (*NEAT1*), *taurine up-regulated 1* (*TUG1*) and *7SK small nuclear RNA* (*RN7SK RNA*) [Santoro et al. 2016]. *NEAT1* promotes the increase of *CXCL8* expression of the gene encoding Interleukin 8 via relocation of SFPQ splicing factor, *TUG1* is a component of the p53 regulatory network and *RN7SK RNA* is involved in regulation of CD4+ T lymphocytes. These lncRNA play an important role in neurodegenerative processes. In the present study we analyze expression profile of lncRNAs in the serum of secondary progressive MS (SP-MS) patients to identify any lncRNAs specifically expressed in progression phase of the disease.

MATERIALS AND METHODS

Patients

We analyzed serum samples from 12 healthy volunteers and 12 SP-MS patients. Inclusion criteria for both groups were age between 18 and 60 years. Exclusion criteria were pregnancy; steroid therapy or infection within 1 month from sample collection and other autoimmune diseases, oncologic disease, or chronic infectious disease. Inclusion criteria for SP-MS patients was diagnosis of SP-MS according to the revised McDonald's criteria [Polman et al. 2011].

RNA extraction

Whole blood samples were collected from participants and serum was separated. Total RNA was extracted from serum using miRNeasy Mini kit (QIAGEN, Germany) according to the manufacturer's instructions..

LncRNA amplification

Extracted RNA was subjected to complementary DNA (cDNA) synthesis using RT2PreAMP cDNA Synthesis Kit (QIAGEN, Germany) according to the manufacturer's instructions. Briefly, total RNA was treated with DNase I for 30 min at 42 °C. cDNA samples were pre-amplified with RT2lncRNAPreAMP Primer Mix that contained specific set of primers to target genes of Human RT2lncRNAInflammatory Response & AutoimmunityPCR Array (QIAGEN, Germany). qRT-PCR was performed using RT2 SYBR® Green qPCR MasterMix (QIAGEN, Germany). The reaction (25 µl) was aliquoted into the wells of RT2lncRNA PCR Array Human Inflammatory Response & Autoimmunity (QIAGEN, Germany) which contains pre-dispensed, laboratory verified, gene-specific primer pairs [Figure 1].

LncRNA data analysis

For data analysis, the CT values were exported to an Excel file and uploaded into the RT2 PCR Array data analysis web portal at https://www.qiagen.com/dataanalysiscenter.

We selected as reference genes *Ribosomal protein, large, P0* (*RPLP0*, NM_001002), *ZNFX1 antisense RNA 1* (*ZFAS1*, NR_003604), and *Growth arrest-specific 5* (*non-protein coding*) (*GAS5*, NR_002578) based on their stable expression across different sample groups evaluated by geNorm [Vandesompele et al. 2002].

The relative fold-change 2−ΔΔCT method was used to determine the relative lncRNAs expression. Fold-change values greater than 1 indicates a positive- or an up-regulation, and the fold-regulation is equal to the fold change. Fold-change values less than −1 indicate a negative or down-regulation, and the fold regulation is the negative inverse of the fold-change. The p values were calculated based on a Student's t test of the replicate 2^ (−ΔCT) values for each gene in the control and patient groups. We plotted the data analysis of web portal with volcano plot, that combines a p value statistical test (y-axis) with the fold regulation change (x-axis) enabling the identification of genes with both large and small expression changes that are statistically significant.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A2ML1-AS1	ABCA11P	AC000120.7	AC007228.9	AC016629.8	AC068196.1	AC104820.2	CEP83-AS1	CROCCP2	CTC-444N24.11	CTC-487M23.5	CTD-3185P2.1
B	DLEU2	EPB41L4A-AS1	ERICH1-AS1	FAM211A-AS1	FGD5-AS1	FGF14-IT1	FLJ31306	FOXN3-AS2	GAS5	GAS5-AS1	GRM5-AS1	HCG11
C	HCG18	HNRNPU-AS1	HOTAIR	HTR4-IT1	IQCF5-AS1	JPX	LINC00094	LINC00116	LINC00293	LINC00324	LINC00338	LINC00421
D	LINC00635	LINC00657	LINC00662	LINC00667	LL22NC03-N27C7.1	LOC653160	LRRC37BP1	MALAT1	MCM3AP-AS1	MEG3	NAV2-AS5	NCBP2-AS2
E	NEAT1	NUTM2A-AS1	OIP5-AS1	PDXDC2P	RMST	RP11-1134I14.8	RP11-282O18.3	RP11-29G8.3	RP11-325K4.3	RP11-363E7.4	RP11-363G2.4	RP11-367N14.3
F	RP11-38P22.2	RP11-399K21.11	RP11-473I1.10	RP11-473M20.16	RP11-498C9.15	RP11-549J18.1	RP11-819C21.1	RP11-84C13.1	RP11-96D1.10	RP1-239B22.5	RP6-24A23.7	SDCBP2-AS1
G	SENP3-EIF4A1	SIK3-IT1	SLC7A11-AS1	SNHG11	SNHG16	SNHG5	SNHG7	TP73-AS1	TUG1	XIST	ZFAS1	ZNRD1-AS1
H	ACTB	B2M	RPLP0	RN7SK	SNORA73A	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure 1. Schematic plate of 84 lncRNAs validated or predicted to regulate the expression of pro-inflammatory and anti-inflammatory genes and microRNA.

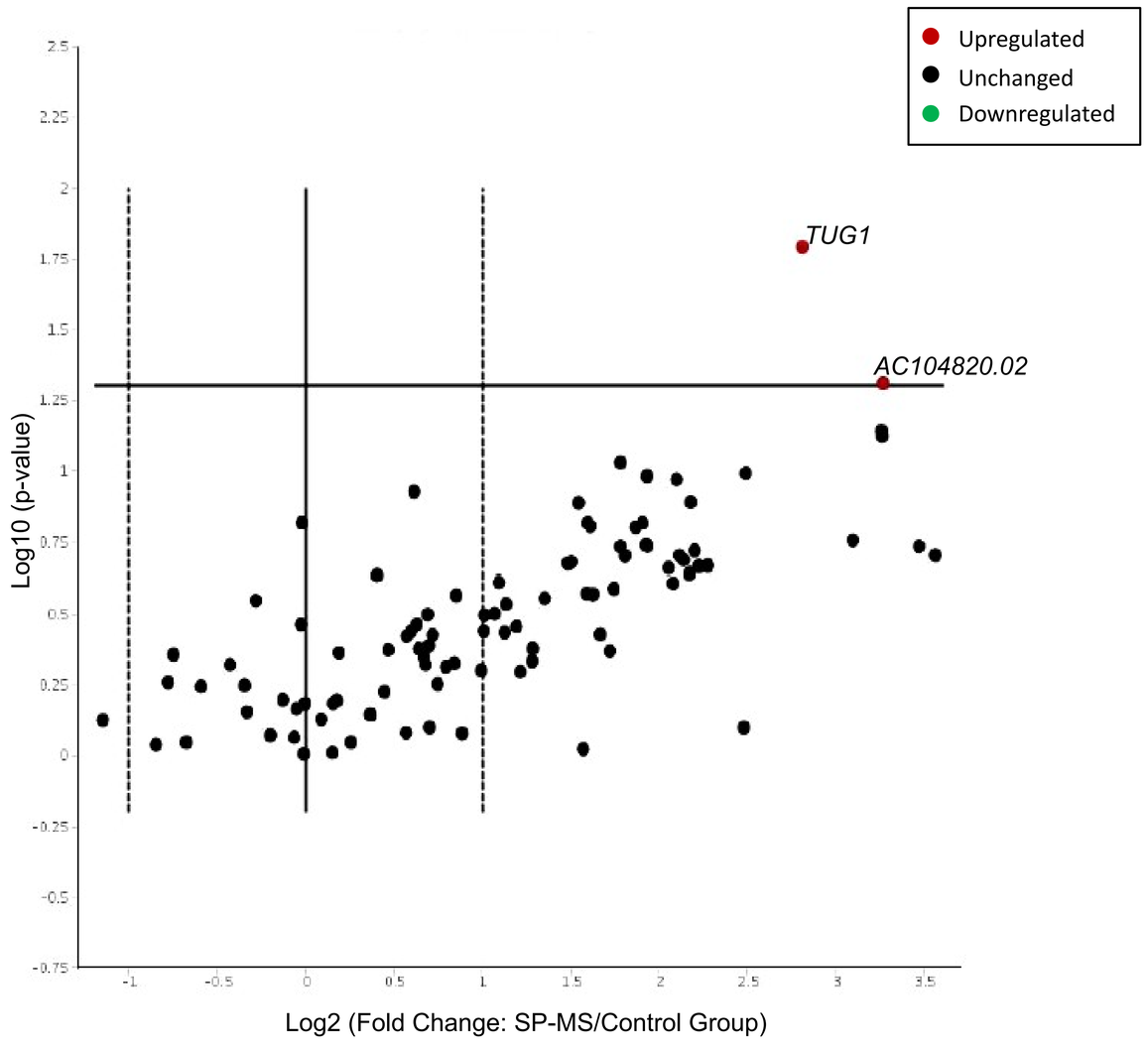

Figure 2. The volcano plot representation of lncRNAs serum levels in SP-MS vs. health controls.
Statistical significance versus fold-change was showed on the y- and x-axes, respectively.
Red circle indicated the up-regulation, green circle the down-regulation, and black circle unchanged regulation.
The values were normalized to the average of expression levels of *RPLP0*, *ZFAS1* and *GAS5*.

RESULTS AND DISCUSSION

In the present study, we analyzed 84 lncRNAs involved in autoimmunity and human inflammatory response, in the serum of SP-MS patients (n = 12) and age-matched controls (n = 12). We identified two lncRNAs up-regulated in SP-MS patients compared to controls: *TUG1*, fold change = 7.00 (p = 0.02) and *AC104820.2*, fold change = 9.60 (p = 0.04) [Figure 2]. *TUG1* promoter contained conserved site that are a direct transcriptional target of putative p53 response. *TUG1* may be a component of p53-regulatory network participating to the apoptotic pathways active in early stages of classical neurodegenerative diseases.
Instead *AC104820.2* is strongly expressed in CD8+ T-cells that are known to be involved in axonal and myelin loss through a possible cytotoxic effect. While *AC104820.2* is specifically expressed in SP-MS, *TUG1* is also upregulated in RR-MS patients [Santoro et al., 2016] but with a minor fold change compared to SP-MS patients. Different MS phenotypes seems to have a specific lncRNA expression profile. Altered epigenetic gene regulation involved in neurodegenerative mechanisms could have an important role in MS pathology. Additional data in larger cohort of MS samples at different stage of the disease are needed to better understand lncRNAs role in MS.

REFERENCES

- Santoro, M. *et al.* Expression Profile of Long Non-Coding RNAs in Serum of Patients with Multiple Sclerosis. J Mol Neurosci. **59**, 18-23 (2016).
· Polman, C.H. *et al*. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol. **69**, 292–302 (2011).
·Vandesompele, J. *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034 (2002).