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A pilot study of lncRNAs expression profile in serum of progressive multiple sclerosis patients

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Abstract. – OBJECTIVE: Multiple Sclerosis (MS) is an inflammatory and neurodegenerative disease that affect both white and gray matter. The relapsing and the eventually progressive course of MS is heterogeneous; thus, a confident long-term prediction of individual prognosis is not possible yet. Recent studies have demonstrated the role of long non-coding RNA (lncRNAs) as potential biomarkers that could provide information to predict disease activity and progression.

PATIENTS AND METHODS: By qRT-PCR, we analysed the lncRNAs expression in the serum of 16 secondary progressive MS (SP-MS), 12 primary progressive (PP-MS) patients and 8 healthy controls.

RESULTS: We found that TUG1 was upregulated in SP-MS, while the comparison of PP-MS vs. controls showed a downregulation of non-protein coding RNA 188 (LRRC75A-AS1) and a significant upregulation of two lncRNAs: long intergenic non-protein coding RNA 293 (LINC00293) and RP11-29G8.3. Moreover, we performed an in-silico analysis using DIANA-LncBase v2 and HMDD v3.0 software, in order to predict the possible interaction of these four lncRNAs with miRNAs. We identified 21 miRNAs prediction targets possibly involved in MS.

CONCLUSIONS: Our data indicate a regulatory function of these lncRNAs in autoimmune and inflammatory processes related to MS suggesting their potential role in progressive MS pathogenesis.

Key Words:
Multiple sclerosis, Long non-coding RNA, Autoimmunity.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease that affects the Central Nervous System (CNS), representing the second leading cause of sustained neurological disability in young people after trauma[1].

Most people with MS present a relapsing remitting (RR-MS) course at onset, characterized by acute relapse (new neurological symptoms lasting more than 24 hours) followed by variable recovery with periods of relative clinical stability in between[2]. After a variable timeframe, RR-MS patients can develop progressive and irreversible neurological disability independent or partially dependent from relapses. The conversion rate to secondary progressive MS (SP-MS) is variable, ranging between 15 and 30% over long-term follow-up[3].

Some patients have a primary progressive (PP-MS) course characterized by a progressive decline in neurological function from disease onset[2].

In the last two decades, RR-MS treatment has changed radically with the introduction of highly effective drugs capable of significantly modifying the natural history of the disease. On the other hand, despite important recent advances, the treatment of progressive MS is still unsatisfactory[3].

Axonal or neuronal loss, demyelination and astrocytic gliosis are hallmarks of MS pathology. Among these neuropathological characteristics, axonal or neuronal loss (referred to as neurodegeneration) is particularly relevant because it is the main underlying mechanism of permanent clinical disability, which accrues predominantly in the progressive forms of the disease[1,3]. However, neurodegeneration actually begins since the earliest stages of the disease process; in addition, it is still possible for patients with a progressive clinical course to have evidence of ongoing inflammatory activity,

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which was previously thought to be exclusive of the RR course[2]. MS pathogenesis and specifically the pathogenic mechanisms underlying disease progression are complex and incompletely understood, but increasing studies underline the crucial role of gene dysregulation in the CNS[2,4].

Therefore, noncoding RNAs (ncRNAs), especially long noncoding RNAs (lncRNA) and micro RNAs (miRNA), have piqued the interest of researchers due to their known functions in the regulation of gene expression as transcriptional, post-transcriptional, translational and epigenetic factors[4].

LncRNAs are a heterogeneous group of ncRNAs classified as 200 nucleotides long transcripts[5].

Unlike mRNAs, lncRNAs lack an open reading frame (ORF) of significant length and have no translational capacity[6].

After being transcribed by RNA polymerase II, lncRNAs undergo transcriptional editing (splicing, polyadenylation and 5' capping) to develop a final stable structure that enables them to interact with other molecules and exert their unique cellular function[6]. Many antisense lncRNAs affect the expression of their partner genes *via* both *cis* and *trans* mechanisms or influencing mRNA stability, while the mechanisms through which non-overlapping lncRNAs regulate gene expression is still largely unknown[5]. Deregulation of lncRNA expression has been involved with the pathogenesis of both autoimmune and neurological diseases[7-9].

In a previous work, the expression profile of lncRNAs was analysed in the serum of RR-MS patients compared to age-matched controls and to patients with idiopathic inflammatory myopathies. This study showed the upregulation of three specific lncRNAs (*RN7SK RNA*, *TUG1* and *NEAT1*) involved in both inflammation and/or neurodegeneration processes[10].

Over the last years, the MS research field has been enriched by many other studies concerning the expression patterns of lncRNAs and their role in the disease pathogenesis, with the ultimate purpose of finding potential clinical applications in MS diagnosis and treatment[4].

Through a wide microarray assay, Zhang et al[11] found the dysregulation of six lncRNAs in peripheral blood mononuclear cells (PBMCs) of RR-MS patients.

Two different studies identified four lncRNAs with putative roles in the regulation of immune response (THRIL, FAS-AS1, PVT1, linc-MAF-4) deregulated in PBMCs of RR-MS patients[12,13].

Sun et al[14] documented the upregulation of the lncRNA Growth arrest-specific 5 (non-protein coding) (GAS5) in amoeboid-shaped microglial cells of MS patients also demonstrating its role in promoting microglial M1 polarization and in demyelination induction.

So far, lncRNAs expression in MS has been mostly investigated in PBMCs and exclusively in patients with the relapsing-remitting form of disease.

The aim of the present study was to explore the expression profile of a specific subset of lncRNA in the serum of PP-MS and SP-MS patients compared to age matched controls.

Patients and Methods

Patients

We collected serum samples from 28 consecutive progressive MS patients (16 SP-MS and 12 PP-MS) and 8 age-matched healthy volunteers (mean age 52.4 ± 8.8). The exclusion criteria were: pregnancy or lactation; steroid therapy or infection within 1 month from sample collection; and other autoimmune, oncologic or chronic infectious diseases.

PP-MS were diagnosed according to the revised McDonald's criteria[15] and SP-MS met clinical diagnostic criteria by Lublin and Reingold[16].

The demographic and clinical parameters of the SP-MS and PP-MS patients were as follows: mean age 56.3 ± 10.5 and 53.1 ± 9.7 years; female sex 68,8% and 50% respectively; mean disease duration 22.8 ± 10.8 and 8.3 ± 4.9 years; mean Kurtzke Expanded Disability Status Scale (EDSS scores) were 6.4 ± 0.9 and 5.0 ± 0.9.

The study was approved by the Local Ethical Committee (Fondazione Policlinico Universitario 'A. Gemelli' IRCCS, Rome, Italy). Written informed consent was obtained from all patients after a detailed explanation of the study's aims and procedures.

LncRNA Analysis in Serum of MS Patients

10 ml of whole blood samples were collected from participants and serum was separated by centrifugation at 3,000 rpm and 4°C for 15 min. Total RNA was extracted from serum using miRNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and subjected to complementary DNA (cDNA) synthesis using RT^2 PreAMP cDNA Synthesis Kit (QIAGEN, Hilden, Germany) for 30 minutes at 42°C. Subsequently, cDNA was pre-amplified

with RT^2 lncRNA PreAMP Primer Mix containing specific primers to target genes of Human RT^2 lncRNA Inflammatory Response & Autoimmunity PCR Array (QIAGEN, Hilden, Germany).

The real-time cycler conditions for pre-amplification on StepOnePlus thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) consisted of holding stage at 95°C for 10 minutes (HotStart DNA Taq Polymerase activation) followed by 12 cycles of each PCR step: (denaturation) 95°C for 15 s and (annealing/extension) 60°C for 2 min.

Primer residues were eliminated by Side Reaction Reducer (QIAGEN, Hilden, Germany) at 37°C for 15 min and inactivation at 95°C for 5 min. qRT-PCR was performed using RT^2 SYBR® Green qPCR MasterMix (QIAGEN, Hilden, Germany). The reaction (25 µl) was aliquoted into the wells of RT^2 lncRNA PCR Array Human Inflammatory Response & Autoimmunity which contains pre-dispensed, laboratory verified and validated gene-specific primer pairs (array #LAHS-004Z, QIAGEN, Hilden, Germany; **Supplementary Table SI**). Each PCR array also includes patented control elements for: genomic DNA contamination detection, RNA sample quality, PCR array reproducibility and RT efficiency (QIAGEN, Hilden, Germany).

Thermal cycling conditions on StepOnePlus thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) consisted of holding stage at 95°C for 10 min followed by 40 cycles of each PCR step: (denaturation) 95°C for 15 s and (annealing/extension) 60°C for 1 min.

For data analysis of results, CT values were exported to an Excel file and uploaded into the RT^2 PCR Array data analysis web portal at https://www.qiagen.com/dataanalysiscenter.

CT values were normalized with three reference genes: Ribosomal protein, large, P0 (RPLP0, NM_001002), ZNFX1 antisense RNA 1 (ZFAS1, NR_003604), and *GAS5* (NR_002578)[10].

Fold change was calculated with ΔΔCT method, in which ΔCT is calculated between gene of interest and an average of reference genes, followed by ΔΔCT calculations [ΔCT(patient)-ΔCT(control)]. Then, $2^{-\Delta\Delta CT}$ formula was used to calculate the fold change. An upregulation was obtained by fold-change values greater than 1 while a downregulation was obtained by fold-change values less than −1.

The *p*-values were calculated with a Student's *t*-test and the data were plotted with volcano plot, that combines a *p*-value statistical test (y-axis) with the fold change (x-axis).

Prediction Analysis For lncRNA-MiRNA Interactions

To predict lncRNA-miRNA interaction we performed an *in-silico* analysis using DIANA-LncBase v2 (www.microrna.gr/LncBase)[17].

The miRNAs, found through DIANA-LncBase v2, were analyzed with Human Micro-RNA Disease Database (HMDD v3.0) (http://www.cuilab.cn/hmdd)[18,19], to assess any possible involvement in MS disease.

Results

Both patient groups were age (p=0.57) and sex (p=0.61) matched with the control group.

In order to evaluate the expression levels of lncRNAs in SP-MS and PP-MS patients *vs.* controls, we analysed 84 lncRNAs, validated or predicted to regulate the expression of pro-inflammatory and anti-inflammatory genes **(Supplementary Table SI)**.

For data analysis we used two criteria: i) the CT cut-off was set to 30 cycles to dismiss those lncRNAs with low expression levels; ii) significant changes (p-value <0.05) were at least two-fold upregulated or downregulated as compared to controls.

Based on these criteria, we identified one lncRNA that was significantly upregulated in SP-MS patients compared to controls: taurine up-regulated 1 (TUG1, NR_002323) fold change = 2.84 (p = 0.012) (Figure 1A).

Moreover, comparing PP-MS *vs.* controls we found a significant downregulation of non-protein coding RNA 188 (LRRC75A-AS1, NR_027158) fold change = -3.73 (p = 0.00003) and a significant upregulation of two lncRNAs: long intergenic non-protein coding RNA 293 (LINC00293, ENST00000518278) fold change = 3.00 (p = 0.016) and RP11-29G8.3 (ENST00000563635) fold change = 2.31 (p = 0.018) (Figure 1B).

Furthermore, using DIANA-LncBase v2 tool several prediction miRNA targets for all four lncRNAs deregulated in both SP-MS and PP-MS patients were identified.

Subsequently, highlighted miRNA targets were analyzed by HMDD v3.0 tool in order to identify those implicated in the pathogenic mechanism of MS. Twenty-one miRNAs were identified (Table I).

Discussion

The present study analysed the expression levels of 84 lncRNAs validated or predicted to regulate

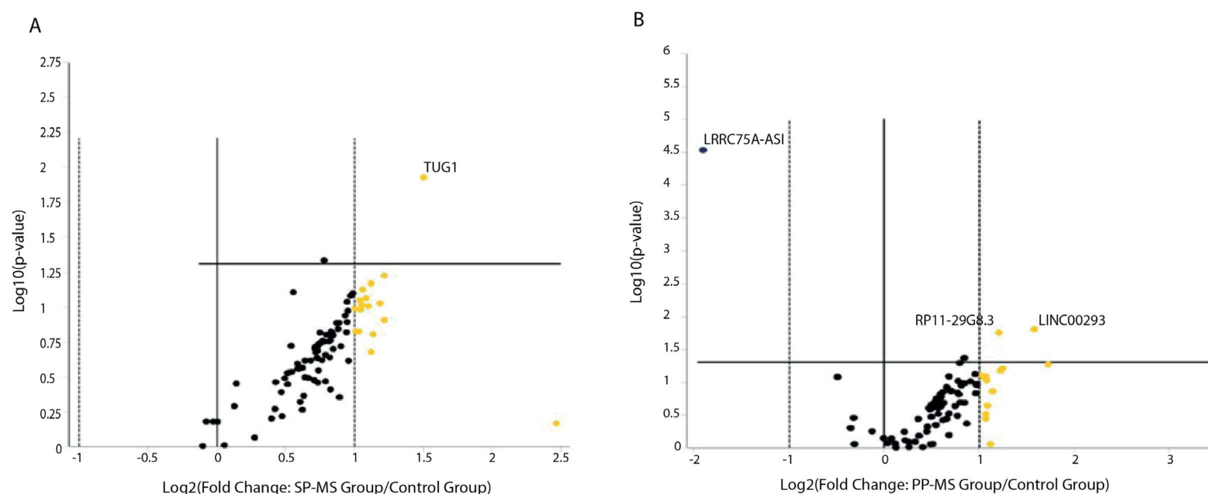

Figure 1. The volcano plot representation of lncRNAs serum levels in SP-MS and PP-MS patients *vs.* health controls. Statistical significance *vs.* fold-change was showed on the y-axes and x-axes, respectively. Yellow circle indicated the upregulation, blue circle the downregulation and black circle unchanged regulation. **A,** SP-MS patients *vs.* control group values were normalized to the average of expression levels of *RPLP0*, *ZFAS1* and *GAS5*. **B,** PP-MS patients *vs.* control group values were normalized to the average of expression levels of *RPLP0*, *ZFAS1* and *GAS5*.

pro-inflammatory and anti-inflammatory genes in serum samples from progressive MS patients.

SP-MS patients showed a significant upregulation of one lncRNA, TUG1 (fold change = 2.84; p = 0.012), compared to controls. TUG1 is a direct downstream target of p53 and a component of p53-regulatory network, that regulates cell cycle progression and the pro-apoptotic pathway active in early stages of neurodegenerative diseases, such as Huntington's disease[10,20].

This result is in agreement with a previous study that showed an upregulation of TUG1 in serum of RR-MS patients[10].

Moreover, comparing PP-MS to controls three deregulated lncRNAs (in all cases fold change >2 and p <0.05) were detected. LRRC75A-AS1 was significantly downregulated while LINC00293 and RP11-29G8.3 were upregulated. At present, there are no literature data indicating a possible involvement of these three lncRNAs in inflammatory or neurodegenerative diseases.

Recent findings demonstrated that many lncRNAs contain predicted miRNA binding sites through which they sequester miRNAs and reduce the pool of available miRNA in the cell[21].

This sponge-like function of lncRNAs determines the negative regulation of miRNA function (process known as competing endogenous RNA – ceRNA – hypothesis) and, by extension, the positive regulation of gene expression[17,21].

Based on these premises and on the wide availability of studies concerning miRNA expression profiles in MS, DIANA-LncBase v2 and HMDD v3.0 tools[17-19] were used to predict the interactions between miRNAs and the deregulated lncRNAs detected in the cohort of SP-MS and PP-MS patients. Twenty-one miRNAs were identified (Table I), whose involvement in MS has already been acknowledged.

Among the 8 miRNAs targeted by TUG1, miR-20a-5p is downregulated in peripheral blood samples of treatment-naïve RR-MS patients. MiR-20a-5p targets include genes involved in p38 MAPK signalling pathway, which plays an essential role in the progression of MS clinical signs by inducing the production of IL-17 from $CD4^+$ Th17 cells and of pro-inflammatory cytokines from $CD4^+$ Th1 cells[22,23]. Considering the well-known role of MAPK pathway in IFN signalling, Ehtesham et al[23] concluded that the pleiotropic effects of IFNβ in people with MS might in part be mediated by this miRNA and that miR-20a-5p levels could serve as a biomarker for the response to therapy.

LRRC75A-AS1 interacts with three MS-related miRNAs, two of which were found to be upregulated in the serum samples of MS patients: miR-22 and miR-326[24,25].

MiR-22 targets the B cell translocation gene 1 (BTG1), an anti-proliferative factor whose downregulation lowers the threshold for microglial apoptosis induced by inflammatory stimuli[24].

MiR-22 also targets estrogen receptor α (ESRα): the subsequent estrogen inhibition signalling in T lymphocytes improves the differentiation of Th1 and Th17 cells and suppresses the protective effect of estrogens against CNS autoimmunity[24].

MiR-326 promotes the Th17-mediated immune response, by suppressing the expression of Ets-1, a known negative regulator of Th17 cells differentiation[26]. MiR-326 is strongly upregulated in active MS lesions too, where it downregulates the expression of CD47 on the membrane of brain-resident cells. Since CD47 is involved in self-recognition processes and protection from phagocytosis, its reduction leads to the release of macrophages from inhibition and subsequent myelin damage[27]. MiR-338 is the only MS-associated miRNA predicted to interact with LINC00293, but its expression pattern in serum samples of MS patients has not been explored yet.

RP11-29G8.3 interacts with 10 MS-related miRNAs, 5 of which have been observed to be deregulated in blood samples of people with MS: miR-200a, miR-141, miR-24-3p, miR-15a and miR-15b.

MiR-15a was found to be downregulated in both peripheral blood samples and $CD4^+$ T cells of MS patients[28]. As already demonstrated in CD4+ T cells, MiR-15a interacts with the pro-apoptotic factor BCL-2 contributing to the reduced apoptosis of autoreactive T cells[29].

Lastly, miR-15b expression levels were found to be downregulated in both serum and PBMCs of people with MS[30,31]. Its targetome includes O-linked N-acetylglucosamine transferase (OGT), whose expression was proven to be increased in PBMCs of MS patients, leading to the glycosilation of NF-κB, which in turn enhances RORγT

Table I. List of interaction between lncRNAs and putative miRNAs.

lncRNA	miRNA	Prediction Score*	PMID
LRRC75A-AS1 (ENST00000475947)	hsa-miR-22-3p	0.843	22231906
	hsa-miR-326	0.893	29043654
	hsa-miR-548ac	0.785	25795118
LINC00293 (ENST00000518278)	hsa-miR-338-3p	0.91	21908875
RP11-29G8.3 (ENST00000563635)	hsa-miR-107	0.586	26943961
	hsa-miR-141-5p	0.812	25938517
	hsa-miR-15b-5p	0.638	24277735
	hsa-miR-16-5p	0.631	24898268
	hsa-miR-200a-5p	0.640	25938517
	hsa-miR-24-3p	0.590	29527713
	hsa-miR-379-3p	0.937	29325906
	hsa-miR-499a-3p	0.613	26305248
	hsa-miR-15a-5p	0.647	22463747
	hsa-miR-29a-5p	0.691	22772450
TUG1 (ENST00000540687)	hsa-miR-106b-5p	-	29194612
	hsa-miR-20a-5p	-	27752929
	hsa-miR-29a-3p	0.941	22772450
	hsa-miR-29b-3p	0.941	29194612
	hsa-miR-30a-5p	0.434	27581464
	hsa-miR-30c-5p	0.428	29551498
	hsa-miR-379-3p	0.395	29325906
	hsa-miR-29c-3p	0.941	23946286

*Prediction score (or miTG score) is a general score for the predicted interaction, the closer to 1, the greater the confidence. The higher miTG score corresponds to a high probability of targeting[17-19].

transcription and the subsequent differentiation of Th17 cells[31].

Conclusions

This pilot study documented, for the first time to the best of our knowledge, the dysregulated expression of four lncRNAs in the serum of patients with SP-MS and PP-MS: TUG1, LRRC75A-AS, LINC00293 and RP11-29G8.3.

Though the present findings require confirmation in larger samples, bioinformatics analysis showed a putative interaction miRNAs-lncRNAs. Additional experiments on the functional and the physiological role of these lncRNAs are needed in order to understand the pathogenic mechanism of MS and to develop personalized pharmacological and rehabilitation therapies for MS patients' treatment.

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Conflict of Interests

All authors have made substantial contributions to this work. All the authors have no actual or potential conflict of interest to disclose regarding this work.

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