Molecular Biology Reports
https://doi.org/10.1007/s11033-019-04614-z

ORIGINAL ARTICLE

Serum miR-30c-5p is a potential biomarker for multiple system atrophy

Annamaria Vallelunga[1] · Tommaso Iannitti[2] · Giovanna Dati[1] · Sabrina Capece[1] · Marco Maugeri[3] · Ersilia Tocci[4] · Marina Picillo[1] · Giampiero Volpe[5] · Autilia Cozzolino[6] · Massimo Squillante[5] · Giulio Cicarelli[6] · Paolo Barone[1] · Maria Teresa Pellecchia[1]

Received: 28 August 2018 / Accepted: 18 January 2019

Abstract
Multiple system atrophy (MSA) is a neurodegenerative disease that belongs to the α synucleinopathies. Clinically, there is an overlap between MSA and Parkinson's disease (PD), especially at the early disease stage. However, these two pathologies differ in terms of disease progression. Currently, no biomarker exists to differentiate MSA from PD. MicroRNAs are non-coding RNAs implicated in gene expression regulation. MiRNAs modulate cellular activity and they control a range of physiological and pathological functions. miRNAs are found in biofluids, such as blood, serum, plasma, saliva, and cerebrospinal fluid. Many groups, including ours, found that circulating miRNAs are differently expressed in blood, plasma, serum and cerebrospinal fluid of PD and MSA patients. In the present study, our primary aim was to determine if serum mir-30-5p and mir-148b-5p can be used as biomarkers for early diagnosis of PD and/or MSA. Our secondary goal was to determine if serum levels of those miRNAs can be correlated with the patients' clinical profile. Using quantitative PCR (qPCR), we evaluated expression levels of miR-30c-5p and miR148b-5p in serum samples from PD (n = 56), MSA (n = 49), and healthy control (n = 50) subjects. We have found that miR-30c-5p is significantly upregulated in MSA if compared with PD and healthy control subjects. Moreover, serum miR-30c-5p levels correlate with disease duration in both MSA and PD. No significant difference was found in miR-148b-5p among MSA, PD and healthy control subjects. Our results suggest a possible role of serum miR-30-5p as a biomarker for diagnosis and progression of MSA.

Keywords Parkinson's disease · MiRNAs · Multiple system atrophy · MiR-30c-5p · Biomarker · Synucleinopathies

Introduction

Parkinson's disease (PD) and multiple system atrophy (MSA) are two neurodegenerative diseases that are part of the family of synucleinopathies, a class of pathologies characterized by accumulation of α-synuclein (α-syn) in the cytoplasm of neurons or glial cells. Many studies have suggested that α-syn has a central role in neural differentiation and synaptic plasticity, but the specific function of α-syn in the brain is unknown [6]. PD is the most common neurodegenerative disease after Alzheimer's disease (AD). The accumulation of α-syn in Lewy bodies in dopaminergic neurons is a specific feature of PD. The Lewy bodies induce death of dopaminergic neurons in several brain areas. The main clinical manifestations are motor symptoms such as bradykinesia, rigidity, and rest tremor; non-motor symptoms are also observed and they include neuropsychiatric dysfunction, cognitive impairment, sleep disorders, olfactory dysfunction, sensory symptoms, and pain [9]. MSA is a rapidly progressive and rare disease. Clinically, it is characterized by a combination of cerebellar impairment autonomic dysfunction, parkinsonism and pyramidal signs.

PD is characterized by accumulations of α-syn in neurons, while MSA is characterized by α-syn accumulation mainly in glial cells in the form of glial cytoplasmic inclusions and, in some circumstances, in neurons [24]. The overlap of symptoms with PD at an early stage may result in misdiagnosis. The differential diagnosis between PD and MSA is based on clinical features, response to dopaminergic treatment and brain imaging findings. Despite growing research efforts, currently, no reliable blood or cerebrospinal fluid (CSF) biomarker exists to discriminate MSA from PD [12].

✉ Annamaria Vallelunga
vallelungaannamaria@gmail.com

Extended author information available on the last page of the article

Published online: 27 February 2019

Evidence suggests that in many neurodegenerative diseases there is a dysregulation of microRNAs (miRNAs). MiRNAs are a group of molecules that regulate the expression of many genes. These non-coding RNAs control gene expression through mRNA degradation or translational repression [4]. Around 70% of known miRNAs are expressed in the brain and functional studies indicate that they are involved in key signaling pathways of brain development such as synaptogenesis, neurite outgrowth, neuronal plasticity, and memory processes [16]. MiRNAs are modulators of cellular activity in several pathological and physiological conditions and they can also be released from the cells and enter into circulatory biofluids such as blood, serum, plasma, saliva, and urine [7]. Several studies have identified many miRNAs specifically deregulated in PD patients [2, 10]. Moreover, different groups, including ours, have found that several circulating miRNAs (cmiRNAs) are differently expressed in the serum samples of MSA subjects compared to PD patients and healthy controls (HC). These cmiRNAs are involved in cell cycle regulation, apoptosis modulation and post-transcriptional modifications, and are strongly expressed in the central nervous system [11, 22].

In a previous study, we found a specific down-regulation of mir-30c-5p in a small sample of PD patients compared with HC and MSA patients. In the same study, we also found that miR-148b-5p was up-regulated in MSA subjects if compared with PD patients [22]. The primary aim of our study was to evaluate if mir-30c-5p and miR-148b-5p are differentially expressed in a larger sample of PD and MSA patients. The secondary aim was to assess whether miR-30c-5p and miR-148b-5p expression correlate with the clinical profile of PD and MSA patients.

Materials and methods

Patients and sample collection

The participants were enrolled at the Center for Neurodegenerative Diseases (CEMAND) at the University of Salerno. We enrolled 3 cohorts of subjects: (1) 49 patients affected by MSA [24 MSA patients with predominant parkinsonism (MSA-P) and 25 MSA patients with predominant cerebellar ataxia (MSA-C)]; (2) 56 PD patients and (3) 50 age-, gender- and race-matched HC with no history of neurological or psychiatric diseases. UPDRS III and UMSARS II were administered to assess disease severity in PD and MSA patients, respectively, at morning on current dopaminergic medication. Participants' demographical and clinical features are shown in Table 1. Exclusion criteria were the presence of genetic forms of PD and other neurological disorders. The present study was approved by the local Ethics Committee at University Hospital S.Giovanni di Dio e Ruggi d`Aragona and all the patients signed an informed consent. For each participant, we obtained blood samples by venipuncture using dry vacutainer tubes (BD Biosciences, Italy). The blood was centrifuged at 3000*g* for 15 min, and the supernatants were collected and stored at − 80 °C.

Table 1 Demographical and clinical features of participants [for age and disease duration, the numbers are expressed as mean ± standard error of the mean (SEM)]

	HC	PD	MSA
Number	50	55	47
Gender (men/women)	23/27	37/19	22/27
Age (years)	64.6 ± 12.5	62.1 ± 6.9	63.2 ± 7.9
Disease duration (months)		26.85 ± 27.47	51.32 ± 30.61
MSA form (P/C)			24/25

RNA isolation, reverse transcription, and quantitative PCR

In order to remove debris or any circulating cell, serum samples were centrifugated for 10′ at 2000 rpm. miRNAs were isolated from 200 µl of serum samples using a total RNA purification kit (Norgen Biotek Corp) and then eluted in 50 µl of elution buffer. RNA was quantified by a fluorometer and spectrophotometer. 2 µl RNA were used for reverse transcription in 10 µl reactions according to the protocol of the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon) (these assays were conducted in triplicate). Then, cDNA was diluted 1:25 and assayed in 10µL PCR reactions. Serum mir-30c-5p and miR-148b-5p were quantified using LNA™ enhanced microRNA assay (Exiqon) according to the manufacturer's instructions. No-template control and no-reverse transcriptase control were run simultaneously. The amplification was performed in a LightCycler®480 Real-Time PCR System (Roche, Germany) in 96 well plates, and the amplification curves were analyzed using the Roche LC software both for determination of Cq (using the 2nd derivative method) and for melting curve analysis.

Data analysis

The accuracy of qPCR-based miRNA expression data depends on the normalization performed using reference genes. MiR-93-5p is recognized as a valid reference miRNA and has been identified to be one of the most stable serum miRNAs. We chose miR-93-5p as reference miRNA to normalize our data. Expression fold changes (fc) were calculated by the $2^{-\Delta\Delta CT}$ method using Genex 6 Enterprise (BioMCC). A one-way ANOVA followed by Bonferroni

post-hoc test was used to compare miRNA expression among PD, MSA and HC groups. All the data was checked for normality using the D'Agostino and Pearson normality test. Since the data was normally distributed, a Pearson's rank correlation coefficient was used to assess the relationships between levels of miRNAs and MSA and PD duration. ROC curve analysis was performed to assess the diagnostic accuracy of miRNAs to discriminate among the groups.

Target prediction

Target prediction of miR-30c-5p was obtained querying the miRTarBase, chosen due to its widespread use and completeness. Only strong mRNA-miRNAs interactions experimentally confirmed by qRT-PCR, luciferase assays and Western Blots were considered. In order to evaluate co-expression relationship among target genes, we used the Search Tool for Retrieval of Interacting Genes (STRING). We extracted the potential target genes with co-expression coefficients > 0.7.

Results

Serum miR-30c was up-regulated in MSA patients if compared with PD and HC

Using miRcury LNA assay, we found that miR-30-5p was significantly up-regulated in MSA patients if compared with PD patients (fc = 1.67; p = 0.0036). Mir-30c-5p was also significantly up-regulated in PD (fc = 1.5; p = 0.0024), and MSA patients if compared with HC (fc = 3.2; p < 0.0001) (Fig. 1). Higher miR-30c levels significantly correlated with longer disease duration in PD (Pearson r = 03855; p = 0.0037) and MSA patients (Pearson r = 0.3527; p = 0.0150). We did not find any correlation with age and disease severity, assessed by UPDRS III and UMSARS scales in PD and MSA, respectively (Fig. 2). We did not observe any significant difference in miR-148b-5p when comparing MSA and PD patients (fc = 0.74; p = 0.89). We did not observe any significant differences in levels of miR-148b when comparing PD patients and HC (fc = 0.6; p = 0.79) and MSA patients and HC (fc = 0.7; p = 0.72).

Diagnostic value of miR-30c-5p

In order to determine the diagnostic accuracy of miR-30c-5p to discriminate between MSA and PD, and MSA and HC, a ROC analysis was performed. The sensitivity of miR-30c-5p to discriminate between MSA and PD was 82%, but the specificity was low (54%). The diagnostic accuracy for discrimination between MSA and controls was good (sensitivity = 0.80; specificity = 0.90).

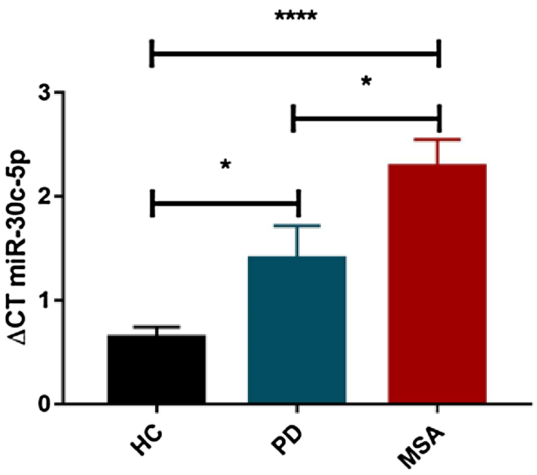

Fig. 1 Relative expression of miRNAs in serum samples from HC, PD, and MSA patients. *p < 0.05 and ****p < 0.0001

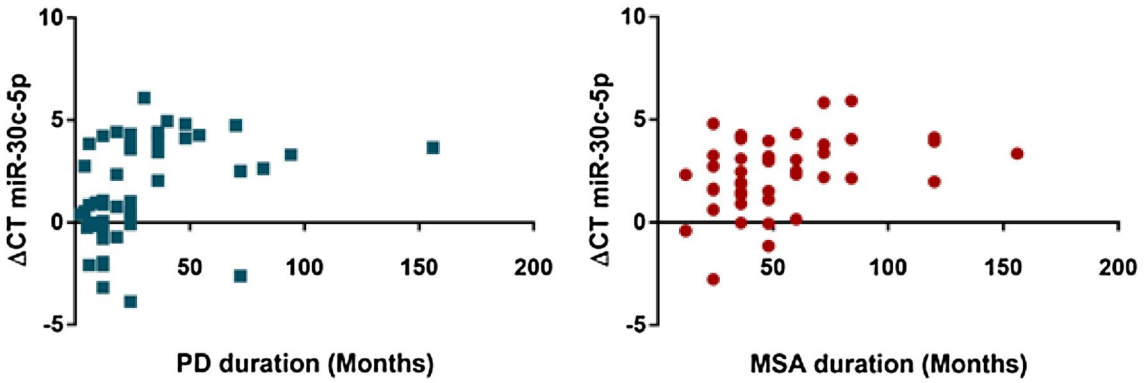

Fig. 2 Correlation between PD and MSA disease duration and miR-30c-5p levels

Target and network analysis of miR-30c-5p

Putative targets of miR-30c-5p were identified by searching the MirTarBase database which contains experimentally validated miRNA target genes. We identified 39 target genes considering only experimentally confirmed strong mRNA-miRNAs interactions. Moreover, we used STRING to visualize the protein interactions (Fig. 3). This network was enriched in many strong interactions among proteins and this result suggests that these proteins work together within their related pathways. Many predicted target genes are involved in neuronal autophagy, mitophagy and regulation of dopaminergic cell death. Specifically, Notch1, Histone deacetylase 4 (HDAC4) and Beclin (BECN1), UBE2I, HSPA4 and DNMT1 are involved in PD and other neurodegenerative diseases.

Discussion

miRNAs have been proposed as reliable biomarkers to diagnose and monitor progression and response to treatment in many neurodegenerative diseases. For the first time, we observed a significant up-regulation of serum miR-30c-5p in MSA patients if compared with PD and HC, suggesting that it may be further studied as a biomarker to differentiate MSA from PD patients. This is in contrast to our previous report, where this up-regulation in miR-30c-5p was not observed possibly due to the smaller sample size in our previous work and differences in terms of disease duration. The positive correlation between miR-30c-5p and disease duration in both PD and MSA groups may suggest a possible role of miR-30c-5p in the progression of synucleinopathies. Using MirTarBase database, we identified 39 experimentally validated miR-30c-5p target genes. In order to establish if there were functional co-expression relationships between predicted target genes, we constructed a network using the STRING software. Enhanced expression of miR-30c-5p could lead to downregulation of the targets predicted [18]. Among the identified gene targets, Notch1, HDAC4, BECN1, UBE2I, HSPA4 and DNMT1 may play an important role in neuronal autophagy and regulation of dopaminergic cell death.

Notch1 has a fundamental role in brain development and adult brain function. The reduction in Notch1 modifies olfaction, synaptic plasticity and memory. Recently, Brai et al. observed that Notch1 is removed and stored

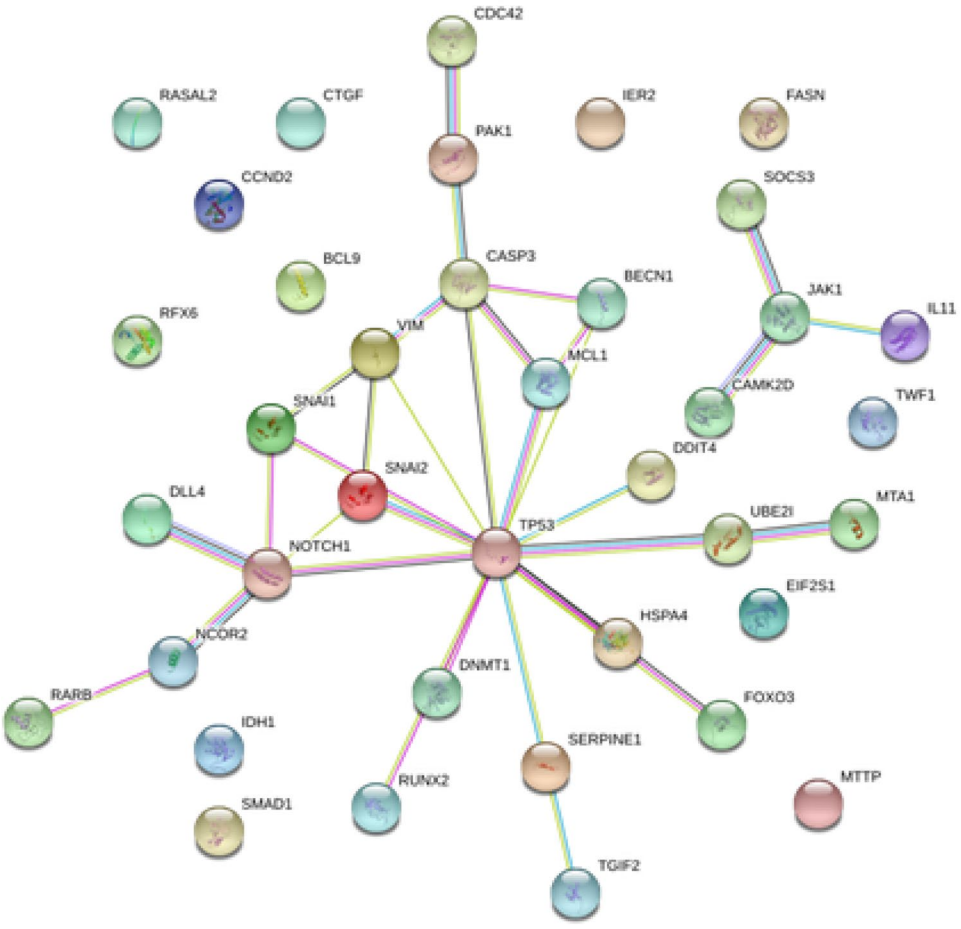

Fig. 3 Network of targets genes of miR-30c-5p. The protein interactions were visualized using STRING software with a co-expression coefficient higher than 0.7

in fibrillary structures in the cortical and hippocampal regions of sporadic AD patients [1]. However, Yoon et al. observed a decrease in Notch1-IC protein stability following Parkin knockdown and they proposed Parkin as a novel regulator of the Notch1 signaling pathway [26]. HDAC4 is a protein highly expressed in the brain. Recently, Wu et al. have observed that intracellular trafficking of HDAC4 is important in the regulation of dopaminergic cell death [25]. Furthermore, in a previous study, Song et al. found that Paraquat-induced histone acetylation was associated with decreased total histone deacetylase activity and HDAC4 protein expression levels in dopaminergic neuronal cells [21]. BECN1 is a pro-autophagic protein that decreases in an age-dependent manner in human brains. Deficiency of BECN1 in cellular and animal models of AD and Huntington's disease was shown to induce disruption of neuronal autophagy, accumulation of mutant huntingtin and amyloid-β and neuronal cell loss [20, 23]. Moreover, Wang et al. found that the overexpression of either wild type or mutated α-synuclein markedly reduced the cytoplasmic levels of BECN1 in PC12 cells, suggesting that BECN1 may contribute to α-synuclein-mediated autophagy [23].

In several studies, BECN1 has been found to interact with PARK2 and PINK1 and those interactions promote autophagy [3, 15]. Finally, Pickford et al. found that BECN1 was decreased in specific brain regions of AD patients [17].

The UBE2I gene encodes for many enzymes of the E2 ubiquitin-conjugating enzyme family, but its function is unknown. Recently, Serpente et al. observed that UBE2I was downregulated in peripheral cells derived from sporadic frontotemporal dementia (FTD) cases compared with controls. Furthermore, in patients with FTD due to C9ORF72 mutation, UBE2I was downregulated [19].

Another interesting target of miR-30c-5p is HSPA4, a member of the heat-shock protein (HSP) family 110 associated with the HSP70 family. HSPA4 is a protein associated with parkin, PINK1 and DJ-1, three genes responsible for the majority of cases of autosomal recessive, early-onset ($\leq$50 years) PD [14]. Microarray data of substantia nigra and putamen from PD patients have shown that HSPA4 was significantly down-regulated in putamen.

DNMT1, an important chromatin regulator, is another target of miR-30c-5p. Recently, Hossein-Nezhad observed that DNMT1 was downregulated in CSF of PD patients [8]. Moreover, a significant reduction in nuclear DNMT1 levels was found in postmortem brain tissue from patients affected by PD and dementia with Lewy bodies as well as in the brains of α-synuclein transgenic mouse models [5].

We suggest that miR-30c-5p may be involved in the pathogenesis of α-synucleinopathies by downregulating its target genes. Serum miR-30c may be useful as a marker of progression of α-synucleinopathies since it is related to disease duration in PD. Unfortunately, we did not assess disease severity of dopaminergic drugs, and this can partially explain the lack of significant correlation between miR-30c and UPDRS and UMSARS in our patients.

No significant difference was found in miR-148b-5p when comparing MSA and PD patients. This is in contrast with our previous report, where miR-148b was found increased in MSA compared to PD patients [22]. This discrepancy could be due to differences in sample size and disease duration. Indeed, the present study enrolled larger samples of patients. Recently, Marques et al. identified a set of miRNAs deregulated in the CSF from PD and MSA patients. In addition, they created three panels based on a combination of CSF miRNAs that were able to differentiate either PD or MSA from controls [13].

Further studies are required to confirm our findings on miR-30c-5p and expand miRNA profiling in PD and MSA. Indeed, serum biomarkers for diagnosis and progression of parkinsonisms are urgently needed and miRNAs are reliable candidates since they are stable molecules which have already been used as biomarkers for other pathologies such as cancer and cardiovascular diseases.

Compliance with ethical standards

Conflict of interest The authors declare that they have no known conflicts of interest in relation to this article.

References

1. Brai E et al (2016) Notch1 hallmarks fibrillary depositions in sporadic Alzheimer's disease. Acta Neuropathol Commun 4(1):64
2. Cardo LF et al (2013) Profile of microRNAs in the plasma of Parkinson's disease patients and healthy controls. J Neurol 260(5):1420–1422
3. Choubey V et al (2014) BECN1 is involved in the initiation of mitophagy: it facilitates PARK2 translocation to mitochondria. Autophagy 10(6):1105–1119
4. De Smaele E, Ferretti E, Gulino A (2010) MicroRNAs as biomarkers for CNS cancer and other disorders. Brain Res 1338:100–111
5. Desplats P et al (2011) α-Synuclein sequesters Dnmt1 from the nucleus: a novel mechanism for epigenetic alterations in Lewy body diseases. J Biol Chem 286(11):9031–9037
6. Galvin JE, Lee VM, Trojanowski JQ (2001) Synucleinopathies: clinical and pathological implications. Arch Neurol 58:186–190
7. Grasso M, Piscopo P, Confaloni A, Denti MA (2014) Circulating miRNAs as biomarkers for neurodegenerative disorders. Molecules 19(5):6891–6910
8. Hossein-nezhad A et al (2016) Transcriptomic profiling of extracellular RNAs present in cerebrospinal fluid identifies differentially expressed transcripts in Parkinson's disease. J Parkinson's Dis 6(1):109–117
9. Jankovic J, Tolosa E (eds) (1998) Parkinson's disease movement disorders, vol 8, pp 159–171

10. Khoo SK et al (2012) Plasma-based circulating microRNA biomarkers for Parkinson's disease. J Parkinson's Dis 2(4):321–331. 12
11. Kume K et al (2018) Serum microRNA expression profiling in patients with multiple system atrophy. Mol Med Rep 17(1):852–860
12. Laurens B et al (2015) Fluid biomarkers in multiple system atrophy: a review of the MSA biomarker initiative. Neurobiol Dis 80:29–41
13. Marques TM et al (2017) MicroRNAs in cerebrospinal fluid as potential biomarkers for Parkinson's disease and multiple system atrophy. Mol Neurobiol 54(10):7736–7745
14. Merwe et al (2015) Evidence for a common biological pathway linking three Parkinson's disease causing genes: parkin, PINK1 and DJ1. Eur J Neurosci 41(9):1113–1125
15. Michiorri S et al (2010) The Parkinson-associated protein PINK1 interacts with Beclin1 and promotes autophagy. Cell Death Differ 17(6):962–974
16. Nadim WD et al (2017) MicroRNAs in neurocognitive dysfunctions: new molecular targets for pharmacological treatments? Curr Neuropharmacol 15(2):260–275
17. Pickford F et al (2008) The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid β accumulation in mice. J Clin Invest 118(6):2190
18. Quévillon Huberdeau M, Simard MJ (2018) A guide to microRNA-mediated gene silencing. FEBS J. https://doi.org/10.1111/febs.14666
19. Serpente M et al (2015) Profiling of ubiquitination pathway genes in peripheral cells from patients with frontotemporal dementia due to C9ORF72 and GRN mutations. Int J Mol Sci 16(1):1385–1394
20. Son JH et al (2012) Neuronal autophagy and neurodegenerative diseases. Exp Mol Med 44(2):89–98
21. Song C et al (2011) Paraquat induces epigenetic changes by promoting histone acetylation in cell culture models of dopaminergic degeneration. Neurotoxicology 32(5):586–595
22. Vallelunga A et al (2014) Identification of circulating microRNAs for the differential diagnosis of Parkinson's disease and multiple system atrophy. Front Cell Neurosci 8:156
23. Wang JD et al (2015) A pivotal role of FOS-mediated BECN1/Beclin 1 upregulation in dopamine D2 and D3 receptor agonistinduced autophagy activation. Autophagy 11(11):2057–2073
24. Wenning GK et al (1994) Clinical features and natural history of multiple system atrophy: an analysis of 100 cases. Brain 117:835–845
25. Wu Q et al (2017) Nuclear accumulation of histone deacetylase 4 (HDAC4) exerts neurotoxicity in models of Parkinson's disease. Mol Neurobiol 54(9):6970–6983
26. Yoon JH et al (2017) Parkin mediates neuroprotection through activation of Notch1 signaling. Neuroreport 28(4):181–186

Affiliations

Annamaria Vallelunga[1] · Tommaso Iannitti[2] · Giovanna Dati[1] · Sabrina Capece[1] · Marco Maugeri[3] · Ersilia Tocci[4] · Marina Picillo[1] · Giampiero Volpe[5] · Autilia Cozzolino[6] · Massimo Squillante[5] · Giulio Cicarelli[6] · Paolo Barone[1] · Maria Teresa Pellecchia[1]

Tommaso Iannitti
tommaso.iannitti@gmail.com

Giovanna Dati
giovanna.dati@hotmail.it

Sabrina Capece
sabrinacapece91@gmail.com

Marco Maugeri
mgrmarco@gmail.com

Ersilia Tocci
lia.tocci@gmail.com

Marina Picillo
picillo.marina@gmail.com

Giampiero Volpe
giampiero.volpe@sangiovannieruggi.it

Autilia Cozzolino
autiliacozzolino77@gmail.com

Massimo Squillante
massysquillante@libero.it

Giulio Cicarelli
cicalio@libero.it

Paolo Barone
pbarone@unisa.it

Maria Teresa Pellecchia
mpellecchia@unisa.it; pellec3@hotmail.com

[1] Neuroscience Section, Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana", University of Salerno, Salerno, Italy

[2] KWS BioTest, Marine View Office Park, Portishead, Somerset BS20 7AW, UK

[3] Department of Rheumatology and Inflammation Research, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[4] Laboratorio Analisi PO Serra San Bruno, ASP Vibo Valentia, Vibo Valentia, Italy

[5] Clinica Neurologica, AOU San Giovanni di Dio e Ruggi d'Aragona, Salerno, Italy

[6] AO San Giuseppe Moscati, Avellino, Italy