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Identification of blood microRNAs associated to Parkinsonĭs disease

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ABSTRACT

The present study demonstrates that blood samples can be used as a source of miRNA identification associated to Parkinson's disease (PD). A set of six differentially expressed microRNAs were identified. They form two groups according to their expression profile in control, non-treated, early-onset and treated Parkinson's disease subjects. While miR-1, miR-22* and miR-29 expression levels allowed to distinguish non-treated PD from healthy subjects, miR-16-2*, miR-26a2* and miR30a differentiated treated from untreated patients. This study is innovative in contributing to the development of effective PD biomarkers.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by resting tremor, rigidity, bradykinesia and loss of postural reflexes. Subjects with PD also can show a variety of non-motor symptoms including cognitive impairment, depression and sleep disorders (Poewe, 2008; Chaudhuri and Odin, 2010).

The molecular mechanism of neurodegeneration in PD is mostly unknown. Pathologically, PD is characterized by the loss of dopaminergic nigral neurons, accompanied by gliosis and the presence of eosinophilic intracytoplasmic inclusions known as Lewy bodies in the remaining substantia nigra pars compacta neurons. Alpha-synuclein is the principal component of filamentous of Lewy bodies, the defining pathological hallmark of PD (Spillantini et al., 1997). It is now recognized that PD is also associated with extensive nondopaminergic pathology (Braak et al., 2003). The etiology of PD is a complex interaction among genes, environment and brain aging. Although in a minority of cases it is due to well-defined genetic causes, a combination of genetic and environmental factors are believed to account for the vast majority of cases. This includes interactions between several genes, modifying effects by susceptibility alleles, the influence of environmental agents on gene expression and their direct impact on the developing and aging brain (Klein and Schlossmacher, 2007).

The use of biomarkers remains investigational in PD. A biomarker is a characteristic that is objectively measured as an indicator of normal or pathogenic biologic processes, or a pharmacological response to a treatment. Biomarkers can help to determine disease pathophysiology and a drug's mechanism of action (Rascol, 2009). The identification of potential biomarkers in PD would be of remarkable value. Currently, there is no reliable biomarker for PD, except in a small minority of patients with a monogenetic form of the disorder, in whom at least the underlying disease trait can be unequivocally determined by mutational analysis. However, even in these patients, the relationship between the biomarker (a mutation detection method) and disease development is complex because of the often reduced and age-dependent penetrance and variable expressivity (Gasser, 2009).

The genetic dissection of common diseases such as PD is a current challenge of medical genomics. Several diseases have genetic components that are not easily detected, in part because loci involved in these conditions may have a small size effects, and these loci may interact among them. This may not be the case for microRNAs, which can regulate a large set of genes from different pathways at the transcriptional and translational levels. Indeed, microRNAs have been used as molecular markers in a series of diseases, including cancer, neurodegenerative disorders and others (Bartels and Tsongalis, 2009; Nelson et al., 2008; Pauley et al., 2009). Recently, it has been proposed that microRNAs (miRNAs) can play a role in PD (Barbato et al., 2009; Eacker et al., 2009; Kim et al., 2007).

MicroRNAs are evolutionarily conserved, small, approximately 22 nucleotides, non-coding RNAs. Most miRNAs are thought to post-transcriptionally inhibit protein coding genes by affecting their translation and/or mRNA stability (Bartel, 2004; Filipowicz

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et al., 2008). The miRNAs bind multiple target RNA species and are associated with many biological functions (Ambros, 2004) like neuronal gene expression (Wu and Xie, 2006) and pathological processes like the development of cancer (Meltzer, 2005), neurodegenerative disorders (Saugstad, 2010; Weinberg and Wood, 2009) and schizophrenia (Perkins et al., 2007).

The present study was designed to evaluate the expression of miRNAs in *de novo* PD (non-treated patients) and the effect of levodopa treatment on miRNA expression. Patients with early manifestation of the disease present a high incidence of affected first-degree relatives, indicating that genetic factors play a role in pathogenesis. Therefore we also evaluated if there were differences in miRNA between early-onset and late-onset PD.

2. Methods

2.1. Subjects and procedures

This study was approved by the local ethics committee and all subjects provided informed consent before entering into the study. Fifteen PD patients attending an outpatient Movement Disorders Clinic of a university hospital in Southern Brazil were included in the study. Eight were untreated PD patients (NT group) with a mean age of 66 years (sd 6.7) and seven were early-onset Parkinson's disease (EOPD) subjects with a mean age of 45 years (sd 8.7). Disease duration was 3.0 (sd 2.6) and 7.2 years (sd 6.6) in the NT and EOPD groups, respectively. Each group was constituted by 50% males.

All patients fulfilled the clinical criteria for PD (the United Kingdom Parkinson's Disease Society Brain Bank clinical diagnostic criteria) (Hughes et al., 1992). Exclusion criteria were as follows: (1) presence of other neurological disease; (2) presence of psychiatric disorder according to a structured clinical interview; (3) Mini-Mental State Examination (MMSE) (Folstein et al., 1975) lower than 24 (Almeida, 1998). The semi-structured interview was used to collect information on demographic variables, disease history and drug record.

The control group (CNT group) was constituted by eight no-PD volunteers with a mean age of 67 years (sd 8.0). These subjects were selected among outpatient clinics and among PD spouses. Control group exclusion criteria were the same as described above.

PD symptoms were evaluated using the Unified Parkinson's Disease Rating Scale (UPDRS) (Fahn and Elton, 1987) and the Hoehn and Yahr modified version (HY) (Hoehn and Yahr, 1967). Most of the NT subjects (62.5%) showed unilateral motor impairment only, corresponding to HY-1stage; while 37.5% of the NT patients had presented bilateral or midline involvement without impairment of balance (HY-2 stage). Distribution of the Hoehn-Yahr Staging among EOPD subjects was as following: HY 1(42.8%), HY-2 (28.6%) and HY-3; corresponding to bilateral disease with mild to moderate disability and postural reflexes impairment (28.6%). Levodopa/carbidopa treatment resulted in motor symptoms improvement evaluated by UPDRS.

Sleep and depressive symptoms were assessed by Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989) and Beck Depression Inventory (BDI) (Beck et al., 1961), respectively. As expected, considering that sleep disturbances are a frequent problem in PD patients (Tandberg et al., 1998), NT group showed worse sleep quality evaluated by PSQI score than control group (mean ± sd: 9.4 ± 4.8 and 4.4 ± 2.5; respectively). PSQI score was 5.7 ± 3.9 in EOPD group. Subjects showed mean BDI scores that were below the cutoff proposed for depression screening (Schrag et al., 2007). No significant differences in MMSE score were found between groups.

Blood samples were taken from all subjects interviewed: *de novo* PD (NT group), early-onset PD (EOPD group), control subjects (CNT group) and from four randomly selected previously untreated PD patients after 97 (±39) days of the levodopa/carbidopa treatment (T group).

2.2. Quantitative real time PCR

Peripheral blood samples were obtained between 5 and 8 PM and immediately mixed with a five-fold volume of Trizol (Invitrogen) for total RNA extraction. Mature microRNA expression was evaluated by quantitative real time PCR (RT-qPCR) of microRNAs by stem-loop RT-PCR, as described by Chen et al. (2005).

Mature miRNA cDNA was synthesized according to the following: 100 ng of total RNA(1:20); 4 μl of stem loop primer mix (Supplementary Table 1) (0.4 μM each); 0.4 μl of M-MLV reverse transcriptase (Promega) 200 U/μl; 4 μl M-MLV reverse transcriptase 5X reaction buffer (Promega) and 2 μl of 5 mM dNTP in a final volume of 20 μl. The synthesis of the cDNA was completed after a series of three incubations: 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min.

The RT-qPCR mix: 10 μl of cDNA (1:30); 0.4 μl of specific microRNAs forward primer (Supplementary Table 1) (20 μM) and universal reverse primer (Supplementary Table 1) (10 μM) primers; 0.4 μl of 5 mM dNTPs; 2 μl of 10X PCR buffer; 1.2 μl of 50 mM $MgCl_2$; 2 μl of 1X SYBR Green® (Molecular probes); 0.05 μl of Platinum Taq DNA polymerase (Invitrogen) to a final volume of 20 μl. SYBR-Green® was used to detect amplification, estimate C_T values and to determine specificity after melting curve analysis. The PCR cycling conditions were standardized to: 94 °C for 5 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 10 s and 72 °C for 15 s. After the main amplification, sample fluorescence were measured at temperatures from 55 to 99 °C with an increasing ramp of 0.1° C in order to obtain the denaturing curve of the amplified products and assure their homogeneity after peak detection and T_m estimation using data obtained from an Applied Biosystems StepOne system.

As an initial step of this study, a PCR array was performed with microRNAs previously reported as potentially associated with both sleep deprivation (Davis et al., 2007) and neurodegenerative disorders (Barbato et al., 2009; Eacker et al., 2009; Kim et al., 2007; Wang et al., 2008). The complete list of the 85 microRNAs used in the PCR array are indicated in Supplementary data Table 2. A set of six microRNAs with more informative expression patterns among each experimental group was chosen. The group was composed of miR-1, miR-22*, miR-29a, miR-16-2*, miR-26a2* and miR-30a, which were selected out of 85 microRNAs present in the initial screening due their differential expression patterns.

2.3. Data analysis

The six selected microRNAs presented an efficiency of amplification in the 0.94–0.98 range among the different cDNA-derived blood samples, as calculated by internal sample exponential amplification analysis (Ramakers et al., 2003). The relative expression was obtained using the $2^{-\Delta Ct}$ method where the Crossing threshold (Ct) values of the target samples are subtracted from the average Ct values of the standard or control samples. The use of $2^{-\Delta Ct}$ is adequate, as the amount of RNA among the different blood samples to produce the cDNAs did not differ significantly and produced similar Ct values among samples for 20 different microRNAs used in the initial screening (data not shown).

Statistical analysis of the relative expression values obtained for each microRNA between pairs of experimental groups were performed by Student's *t*-test implemented in SPSS Statistics 17 software. In order to compare the expression levels among the four experimental groups, the Waller–Duncan and Tukey HSD tests were performed with SPSS 17, with identical group discrimination and similar probability values.

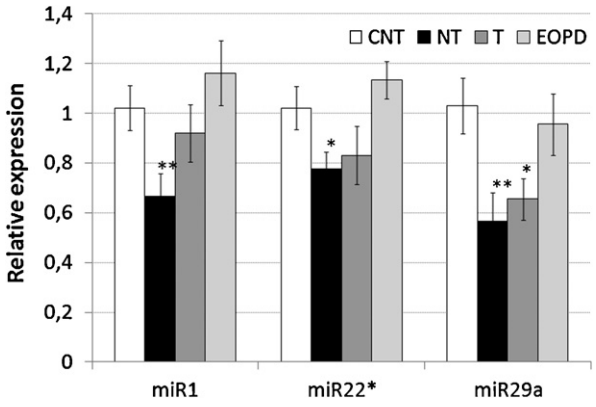

Fig. 1. Relative expressions of miR-1, miR-22* and miR-29a among the patient groups. Mean (± standard deviation) miRNA relative expression in blood of the control (CNT), *de novo* PD (NT), treated PD (T) and early-onset PD (EOPD). Mir1: lower expression in NT than CNT and EOPD; miR-22*: a trend to lower expression in NT than CNT; miR-29a: lower expression in NT (statistically significant) and T (trend) than the CNT group. Waller–Duncan and Tukey HSD tests; double asterisks indicate $p < 0.05$, single asterisks indicates $p = 0.07$.

The target microRNAs that have the potential binding sites for individual microRNAs were identified by searching them on public databases endowed with prediction algorithms present in miRBase (Mendes et al., 2009).

3. Results and discussion

A set of three miRNAs, miR-1, miR-22* and miR-29a, showed reduced profiles of relative expression in the *de novo* PD (NT) and treated groups (T) when compared to control subjects (CNT) and the early-onset PD group (EOPD) (Fig. 1). NT group had significantly lower miR-1 expression than the CNT and EOPD groups ($p = 0.031$ and $p = 0.015$, respectively). In addition, miR-1 expression was not statistically different between the T group, which was constituted by NT patients after levodopa treatment and CNT subjects. It suggests that levodopa treatment can play a role in restoring normal miR-1 relative expression levels. *De novo* PD patients also showed low relative miR-22* expression. In comparison with the CNT group, NT subjects showed a trend toward low miR-22* expression ($p = 0.067$). Considering miR-29a, the NT group showed lower miR-29a expression than the CNT group, and levodopa treatment did not significantly change this expression. Interestingly, low miR-29a expression was maintained after treatment; the T group showed a trend to lower expression than the CNT group.

A second set of three miRNAs, miR-16-2*, miR-26a2* and miR-30a, presented no statistical differences in their relative expressions between the CNT and NT groups (Fig. 2A). These same miRNAs showed a 50% increase in their relative expression in the groups constituted by PD patients taking dopaminergic drugs, EOPD compared to CNT group (Fig. 2B), while only miR-16-2* and miR-26a2* were increased in treated individuals (T) compared to the NT group (Fig. 2C).

Examples of differential miRNA expression have been shown in patients and animal models of neurodegenerative disorders (Roshan et al., 2009). In a mouse model of PD, miR-133b was found to be downregulated (Kim et al., 2007), and analyses on post-mortem tissue showed a decreased expression of miR-133b in the midbrain of patients with PD. However, in our study using blood samples, this miRNA did not show altered expression.

The miRNAs can act at many stages to trigger the production or degradation of toxic proteins, and changes in these steps may have a role in the neurodegenerative process (Eacker et al., 2009). Alpha-synuclein accumulation can interfere with α-tubulin solubility and distribution, leading to neuronal dysfunction. Alpha-

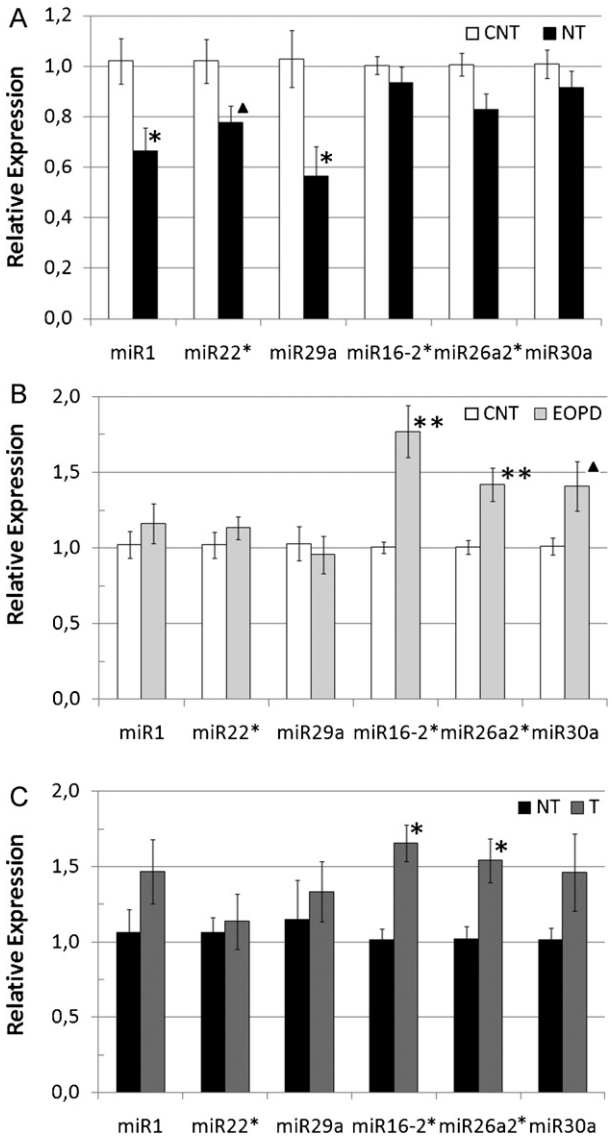

Fig. 2. Pairwise analysis of the set of six miRNAs: miR-1, miR-22*, miR-29a, miR-16-2*, miR-26a2* and miR-30a. Data represent mean (± standard deviation) of miRNA relative expression. (A) *De novo* PD group (NT) showed lower miR1, miR-22* and miR-29a relative expression than the control group (CNT); NT and CNT presented no statistical difference in miR16-2, miR-26a2* and miR30a; Student's *t* test, asterisks indicate $p < 0.05$, triangle indicates $p = 0.007$. (B) Higher miR16-2* and miR26a2* relative expression occurred in early–onset Parkinson's disease (EOPD) than the CNT group; Student's *t* test, double asterisks indicate $p < 0.01$, triangle indicates $p = 0.07$. (C) Treated group (T) showed higher miR-16-2* and miR-26a2* than the NT group; Student's *t* test, asterisks indicate $p < 0.05$.

synuclein overexpression impairs microtubule – dependent traffic, (Lee et al., 2006) and in its aggregated forms, plays a critical role in the pathogenesis of PD. Tubulin polymerization-promoting protein (TPPP/p25) interacts with the tubulin/microtubule system *in vitro* and *in vivo*, and is enriched in human pathological brain inclusions. The primary intracellular target of TPPP/p25 is tubulin/microtubules (Hlavanda et al., 2002) and abnormal TPPP/p25 accumulation has been associated with alpha-synuclein aggregates. It has been proposed that upregulated TPPP/p25 could be a specific marker for pathological conditions associated with abundant aggregation of alpha-synuclein, such as PD (Kovács et al., 2004; Kovács et al., 2007). It should be noted that TPPP/p25 is a target of miR-1, which, in the present study, showed lower expression levels in *de novo* PD when compared with healthy subjects.

In addition to TPPP/p25, clathrin heavy chain (CHC) is also a target of miR-1. Studies have indicated that clathrin may be involved in mediating microglial endocytosis of aggregated alpha-synuclein and associated with microglial activation (Liu et al., 2007). Knockdown of CHC by small interfering RNAs inhibited both constitutive and protein kinase C-mediated internalization of the dopamine transporter (DAT) (Sorkina et al., 2005). Peripheral blood lymphocytes also express many neurotransmitters and neuropeptide receptors. Previous studies have shown that blood samples from PD patients present increased expression of CLTB (Scherzer et al., 2007) and dopamine receptors on lymphocytes (Barbanti et al., 1999).

Considering the current study data, it should be noted that dopamine transport regulation in PD patients is related to miR-1 and mir-30a. The former is associated with CHC properties, as described above, and miR-30a has SLC6A3 (solute carrier family 6 – neurotransmitter transporter – dopamine) as a potential target. The plasma membrane dopamine transporter (DAT) present at the cell surface is regulated by the rate of DAT internalization to endosomes and DAT recycling back to the plasma membrane. DAT is expressed in presynaptic terminals of substantia nigra dopaminergic neurons, where it mediates the re-uptake of synaptic-released dopamine. Alpha-synuclein acts in the modulation of DAT functions by regulating synaptic tone of dopamine (Sidhu et al., 2004).

Moreover, miR-30a may also target fibroblast growth factor (FGF20), which is targeted by miR-16-2*. In this study, both miR-30a and miR-16-2* showed the highest expression in PD subjects after levodopa treatment. A previous study identified a point mutation in PD patients in the 3′UTR of FGF20 that disrupts the miR-433 binding site (Wang et al., 2008). Expression of miR-433 was also analyzed but did not show differences among different groups (data not shown).

Like miR-16-2*, our data demonstrated that PD subjects after levodopa treatment have higher miR-26a2* expression than the *de novo* PD group. miR-26a2* has a tyrosinase precursor gene as one of its potential targets. Another potential target of miR-26a2* is the glutamate receptor 1 precursor (GRIA1), as with miR-30a. Studies have demonstrated that glutamatergic regulation can take place in PD symptoms and treatment (Calón et al., 2003).

The assembly of miR-16-2*, miR-26a2* and miR30a presented higher expression in treated PD (Fig. 3A). The treatment effects were evaluated in two groups: T and EOPD. These miRNAs showed the highest expression in the EOPD group. It should be noted that the EOPD group consisted of patients that were submitted to more than one antiparkinsonian drug, and they had a longer disease duration than the T group. When miR-16-2*, miR-26a2* and miR-30a were analyzed together, we identified that they were expressed significantly higher in both treated groups (EOPD and T groups) than in the CNT and NT groups. A statistical difference was not observed when this analysis was performed between the EOPD and T groups, reinforcing the potential role of the antiparkinsonian drug in these miRNAs expressions.

The miR-1, miR-22* and miR-29a expression levels evaluated together showed that the EOPD group had higher expression levels than the NT and T groups (Fig. 3B). It should be noted that distinct mechanisms can underlie these results. There are some additional features implicated in these data, including that the EOPD and T groups received distinct antiparkinsonian drugs, whose mechanism of action, as well the treatment duration, could exert interference in miRNA expression. Considering the EOPD and NT groups, it should be noted that some group characteristics like disease duration and EOPD pathophysiology may contribute to these results.

Relative expression of miR-1, miR-22* and miR-29a analyzed together did not show a significant distinction between the NT and T groups. Each of these miRNAs (miR-1, miR-22* and miR-29a) showed lower expressions in the NT group compared to the CNT

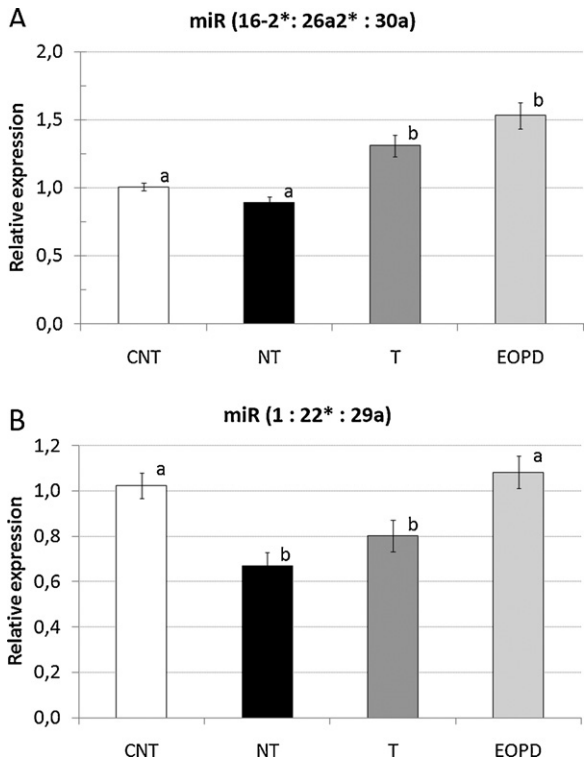

Fig. 3. Grouped miRNA relative expression analysis. (A) Altogether, miR-16-2*, miR-26a2* and miR-30a relative expression analysis showed a significant difference between treated (T and EOPD) and untreated groups (B) assemblage of miR-1, miR-22* and miR-29a expression showed lower expression in *de novo* PD (NT) than the control (CNT) and early–onset Parkinson's disease (EOPD) groups. Waller–Duncan and Tukey HSD tests; different letters indicate statistically significant differences between groups, $p < 0.05$.

group, indicating a possible regulatory mechanism among their targets. Some miR-1 targets have been mentioned previously in this text. Concerning miR-22*, it is appropriate to remark on two of its potential targets: tumor suppressor p53-binding protein 2 (TP53BP2) and GRIA. As cited above, GRIA is also targeted by miR-26a2* and miR-30a.

According to the present data, miR-29a was the only miRNA evaluated where the decreased expression observed in the NT group was maintained after levodopa treatment (Fig. 1). These results allow us to suggest that (i) mir-29a is associated with PD, as demonstrated by the relative expression difference between the NT and control groups, and that (ii) the lower miR-29a expression in PD should not be directly related to the occurrence of motor symptom changes because the decreased miR-29a expression was maintained despite motor symptom improvement. This maintained low expression pattern evokes special interest in terms of miR-29a having a role in PD.

Cell division cycle 42 (CDC42), which is related with septin, is targeted by miR-29a. Recently, postmortem *substantia nigra* of a PD study described the presence of increased levels of septin4 and the parallel increase of septin4 and alpha-synuclein in PD (Shehadeh et al., 2008). Curiously, the expression of miR-29a has also a decreased expression level in Alzheimer's disease, in association with increased BACE1/beta-secretase expression and neuron navigator (Hébert et al., 2008; Shioya et al., 2010).

Considering the neurodegenerative process and cell damage occurrence in PD, some miRNAs evaluated in the present study also have trophic factors as their targets. This can be understood, in one way, as a kind of compensatory mechanism target by some miRNAs. Conversely, the reduction of these trophic factors by distinct features, such as aging, may exacerbate the neurodegenerative

process. It must be considered that brain-derived neurotrophic factor's (BDNF) ability to protect neuronal activity can be affected by aging (Sohrabji and Lewis, 2006). Animal models have shown that increased BDNF expression following striatal damage can be beneficial to dopaminergic neurons; however, these changes in BDNF expression may be lost with age (Batchelor et al., 1999; Collier et al., 2005).

BDNF has been identified as a potential target of miR-1. It is present in neurons, glia and peripheral immune cells. BDNF promotes the survival of dopaminergic neurons and protects them from toxin-induced damage *in vitro* (Murer et al., 2001). BDNF levels are increased in PD cerebral spinal fluid when compared with normal subjects (Salehi and Mashayeki, 2009). This BDNF elevation in CSF may be caused by an increased generation of glial cells resulting from brain damage (Knott et al., 2002). Production of BDNF by glial cells in PD patients may represent an active response to neurodegeneration (Salehi and Mashayeki, 2009). The reduced levels of miR-1, detected in non-treated PD subjects, are in agreement with an increase in BDNF mRNAs and the previously described BDNF protein levels.

Insulin-like-growth factor-1 (IGF-1) is also linked to the preservation and homeostasis of the nervous system. MiR-1, miR-29a and miR-30 all target IGF-1. Serum and brain IGF-1 levels change in several human neurodegenerative diseases (Busiguina et al., 2000). Recently, a study found that blood IGF-1 levels, although statistically non-significant, were mildly elevated in treated PD patients in comparison with controls (Tuncel et al., 2009).

We have shown miRNA expression levels from peripheral blood sources and discussed some potential miRNA targets that could be related to PD pathophysiology. Some limitations should be kept in mind considering the results of this study, like the small sample size and properties of miRNA. A single miRNA can target many transcripts and a single transcript can be targeted by many miRNAs, transcripts can present differences in tissue distribution. We are concerned that the present results were obtained from blood, and that these miRNAs may show different expression patterns in brain tissue. Moreover, these differences in miRNA expression may even occur among distinct brain areas (Davis et al., 2007; Kim et al., 2007). The extent of miRNA expression pattern correlation between peripheral blood and different brain structures should be evaluated in future research.

4. Conclusion

MiR-1, miR-22* and miR-29a showed low expressions in PD blood samples. Altogether, this miRNAs expression analysis showed a significant distinction between the NT and control group. MiR-16-2*, miR-26a2* and miR30a expression levels allowed us to distinguish treated from untreated patients. Despite the cellular functions of many human miRNAs remaining unknown and the complexity of Parkinson's disease, we assume that further miRNA studies will contribute to PD biomarker development. In addition, peripheral blood as a source of this information could facilitate miRNA studies in humans, as well their future clinical applicability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2011.01.023.

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