Parkinsonism and Related Disorders 33 (2016) 115–121

Contents lists available at ScienceDirect

Parkinsonism and Related Disorders

journal homepage: www.elsevier.com/locate/parkreldis

Parkinson's disease: *SNCA*-, *PARK2*-, *and LRRK2*- targeting microRNAs elevated in cingulate gyrus

Roman Tatura [a], Theo Kraus [b], Armin Giese [b], Thomas Arzberger [b, c], Malte Buchholz [d], Günter Höglinger [e], Ulrich Müller [a, *]

[a] Institute of Human Genetics, Justus-Liebig-University, Schlangenzahl 14, Gießen, Germany
[b] Center for Neuropathology and Prion Research (ZNP), Ludwig Maximilians-University Munich, Feodor-Lynen-Str. 23, München, Germany
[c] Department of Psychiatry and Psychotherapy, Ludwig-Maximilians-University Munich, Nussbaumstraße 7, Munich, Germany
[d] Clinic for Gastroenterology, Endocrinology and Metabolism, Philipps-University, Baldingerstrasse, Marburg, Germany
[e] Department of Neurology, Technical University of Munich and German Center for Neurodegenerative Diseases e.V. (DZNE) Munich, Feodor-Lynen Str. 17, Munich, Germany

ARTICLE INFO

Article history:
Received 13 July 2016
Received in revised form
23 September 2016
Accepted 26 September 2016

Keywords:
Parkinson's disease
TaqMan
miR-144
miR-199b
miR-221
miR-488
miR-544
SNCA
PARK2
LRRK2

ABSTRACT

Introduction: In order to better understand the role of epigenetic influences in the etiology of Parkinson's disease (PD), we studied the expression of microRNAs in gyri cinguli of patients and controls.
Methods: Expression profiling of 744 well-characterized microRNAs in gyri cinguli from patients and controls using TaqMan array microRNA cards. Verification of significantly dysregulated microRNAs by SYBR Green qRT-PCR.
Results: First screen by TaqMan array identified 43 microRNAs that were upregulated in gyri cinguli from patients. Of those microRNAs, 13 are predicted to regulate at least one of six genes mutated in monogenic forms of PD (*DJ-1*, *PARK2*, *PINK1*, *LRRK2*, *SNCA*, and *HTRA2*). Five of these 13 microRNAs (*-144*, *-199b*, *-221*, *-488*, *-544*) were also found upregulated by SYBR Green qRT-PCR and are predicted to regulate either *SNCA*, *PARK2, LRRK2* or combinations thereof. Consistently, expression of *SNCA, PARK2, and LRRK2* was reduced in patients. An additional 5 out of ten potential target genes tested were downregulated. These are *DRAM* (DNA damage regulated autophagy modulator 1), predicted to be regulated by *miR-144*, *EVC* (Ellis Van Creveld Protein) by *miR-221*, *ZNF440* (Zinc Finger Protein 440) by *miR-199b*, *MTFMT* (Mitochondrial Methionyl-tRNA Formyltransferase) by *miR-488* and *XIRP2* (Xin Actin Binding Repeat Containing) possibly controlled by *miR-544a*.
Conclusion: The study identified five microRNAs that play a role in the etiology of Parkinson's disease likely by modifying expression of *SNCA*, *PARK2*, *LRRK2* and additional genes required for normal cellular function.

1. Introduction

Most (ca. 90%) cases of Parkinson's disease (PD) are sporadic and have a multifactorial etiology [1]. Various environmental factors, genetic, and epigenetic variants contribute to disease. Environmental factors include insecticides and herbicides [2]. Genetic risk variants have been identified at 24 loci, including alpha-synuclein - encoding *SNCA* and dardarin – encoding *LRRK2* [3]. Epigenetic changes described so far are dysmethylation at several loci in brain DNA of PD patients. In addition there are several reports of dysregulation of microRNAs (miRs) [4].

PD is thought to develop when environmental factors that influence gene regulation e.g. by epigenetic effects coincide with genetic risk variants over an extended period of time. This is consistent with the well-established age-dependent increase in the prevalence of the disorder. While less than 0.1% are affected among persons <60 years of age, prevalence increases to 2–3% in those older than 80 years [5].

Among potential epigenetic risk factors miRs are particularly interesting since individual miRs are involved in the regulation of multiple genes (commonly >100). miRs are small (commonly 22 nucleotides) non-translated RNAs encoded by distinct genes. miR

* Corresponding author. Institut für Humangenetik, Schlangenzahl 14, 35392 Giessen, Germany.
E-mail address: ulrich.mueller@med.uni-giessen.de (U. Müller).

http://dx.doi.org/10.1016/j.parkreldis.2016.09.028
1353-8020/

genes are frequently located in anti-sense direction of coding genes or in introns. Well over 5,000 different miR genes have been identified in the human genome (http://www.mirbase.org). Primary transcripts (pri-miRs) of miR genes are processed into approximately 70-nucleotide stem loop precursor miRNAs (pre-RNAs) by the intranuclear enzyme Drosha. They are exported into the cytoplasm and further cleaved by the ribonuclease Dicer to form mature miRs. Mature miRs regulate gene activity by forming short stretches of double stranded RNAs with target RNAs. This reduces gene activity by either interfering with translation, destabilization or preferential cleavage of target RNAs [6].

In order to learn more about a possible role of miRs in the etiology of PD we studied expression levels of 762 well characterized miRs in gyri cinguli of brains of PD patients and controls.

2. Methods

2.1. Brain samples

Anterior cingulate gyri from 22 Caucasian neuropathologically confirmed PD patients (age: 73.9 years ± 6.9; 54% female/46% male; post-mortem interval (PMI) 30.6 ± 17.4 h) and from 10 Caucasian controls (age: 65.7 ± 10.9 years; 60% female/40% male; PMI 29.4 ± 19.9 h) were provided by the Neurobank Munich, Ludwig-Maximilians-University Munich, Germany to which they had been donated for research. RNA extracted from these tissue samples was used for TaqMan array analysis, SYBR Green (SG) validation, and analysis of target genes. Staging of the cases was done according to McKeith et al. [7]. We distinguished three stages, the „brain stem" (4 cases), the „limbic" (8 cases), and the „neocortical" type (10 cases) of PD. Affection status of the gyrus cinguli is minimal at the brain stem, more severe at the limbic, and most pronounced at the neocortical stage.

2.2. Sample preparation

Total RNA was extracted from tissues using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Concentration and purity of RNA were tested on a GeneQuant II Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). RNA integrity was checked by agarose gel electrophoresis.

2.3. TaqMan miR array and SYBR-green (SG) qRT-PCR

These analyses were done as described before [8]. In brief, quantification of 744 well-characterized human miRs was performed using TaqMan Array MicroRNA cards (pools A and B, Thermo Fisher Scientific, Waltham, USA; Excel file with plate content: https://tools.thermofisher.com/content/sfs/brochures/megaplex-pools-array-card-content.xlsx). 150 ng of total RNA were used for cDNA synthesis and pre-amplification. Quantitative real-time PCR (qRT-PCR) was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Waltham, USA). Data analysis and generation of volcano plots were performed by Expression Suite Software V1.0.3 (Life Technologies, Carlsbad, USA). This software takes into account and corrects for multiple testing of samples. miR expression was normalized relative to endogenously expressed U6 snRNA.

miRs that were dysregulated in the first screen were further analyzed using the NCode™ miRNA First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, USA) and SG qRT-PCR. 1 μg of total RNA was treated with poly A polymerase and subsequently reverse transcribed according to the manufacturer's instructions. Analysis of miR expression was performed on a CFX384 Touch (Bio-Rad, Hercules, USA) with miR-specific primers (Suppl. Table 1). For data analysis CFX Manager 3.1 (Bio-Rad) and Microsoft Excel were used. Validation experiments were repeated three times.

2.4. Expression analysis of potential target genes

Expression of target genes was studied by SG qRT-PCR. Potential target genes were selected from databases miRanda (http://www.microrna.org) and Targetscan (http://www.targetscan.org). Oligonucleotides for qRT-PCR were designed using Primer3-Software version 4.0.0 (http://primer3.ut.ee). Expression of target genes was analyzed with a CFX384 Touch (Bio-Rad) and data was analyzed using CFX Manager 3.1 (Bio-Rad) and Microsoft Excel. Target gene expression was normalized relative to *EIF4A2* and *CYC1* expression. Experiments were repeated three times.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Comparisons between groups were done by unpaired t tests (two tailed, confidence interval 95%). The following p values were considered statistically significant: $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

3. Results

3.1. TaqMan miR arrays identify 43 dysregulated miRs

The volcano blots of Fig. 1 give the results of TaqMan array profiling of 744 miRs in gyri cinguli of 22 PD patients and of 10 controls (panels A & B).

Of these miRs, 43 were significantly ($p < 0.05$) upregulated in brains of patients as compared to controls. The same result was obtained when only same sex samples were compared (not shown). The upregulated genes, their fold-change expression and p-values are listed in Suppl. Table 2. miRs downregulated in PD did not reach significance. However, downregulation of two miRs, i.e. *miR-623* ($p = 0.054$) and *miR-1251* ($p = 0.071$) was almost significant.

We analyzed the 43 upregulated miRs in databases (miRanda and Targetscan) for a potential role in the regulation of several genes implicated in the etiology of PD. From numerous genes implicated in monogenic forms of PD we selected *DJ-1*, *PARK2*, *PINK1*, *LRRK2*, *SNCA*, and *HTRA2* the role of which is soundly established. 13 miRs were found to target at least one of these six genes. *miR-544a* might influence expression of *DJ-1*; *miR*-199b, *miR*-488, *miR-221*, and *miR-543* might modify expression of *PARK2*; *miR-30d* could be involved in regulation of *PINK1*; *miR-23a*, *miR-144*, and *miR-543* might regulate *LRRK2*; *miR-23a*, *miR-29b1*, *miR-488*, *miR-7*, *miR-221*, *miR-144*, and *miR-17* appear to influence *SNCA* expression and *miR-424*, *miR-17*, and *miR-145* influence the expression of *HTRA2*. Mutations in *DJ-1*, *PARK2*, and *PINK1* cause autosomal recessive and mutations in *LRRK2* and *SNCA* autosomal dominant forms of PD [9]. *HTRA2* is a down-stream gene of *PINK1* and autosomal dominant mutations were detected in PD patients [10]. In addition SNPs at loci *LRRK2* and *SNCA* predispose to sporadic PD.

3.2. Validation of upregulation of miRs potentially relevant to PD

We performed SG qRT-PCR for validation of overexpression of those 13 miRs potentially involved in the regulation of genes known to play a role in the etiology of PD. As shown in Fig. 2 *miRs -144, -199b, -221, -488*, and *-544* were significantly upregulated. Upregulation of *miRs -17, -23a, -29b1, -30d, and -424* could not be confirmed by SG qRT-PCR. Three *miR*s (7, 145, 543) found