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Research Paper

The Parkinson's disease gene product DJ-1 modulates miR-221 to promote neuronal survival against oxidative stress

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ABSTRACT

DJ-1 is a highly conserved protein that protects neurons against oxidative stress and whose loss of function mutations are linked to recessively inherited Parkinson's disease (PD). While a number of signaling pathways have been shown to be regulated by DJ-1, its role in controlling cell survival through non-coding RNAs remains poorly understood. Here, using a microarray screen, we found that knocking down DJ-1 in human neuroblastoma cells results in down-regulation of microRNA-221 (miR-221). This is one of the most abundant miRNAs in the human brain and promotes neurite outgrowth and neuronal differentiation. Yet the molecular mechanism linking miR-221 to genetic forms of PD has not been studied. Consistent with the microarray data, miR-221 expression is also decreased in *DJ-1*$^{-/-}$ mouse brains. Re-introduction of wild-type DJ-1, but not its PD-linked pathogenic M26I mutant, restores miR-221 expression. Notably, over-expression of miR-221 is protective against 1-methyl-4-phenylpyridinium (MPP^+)-induced cell death, while inhibition of endogenous miR-221 sensitizes cells to this toxin. Additionally, miR-221 down-regulates the expression of several pro-apoptotic proteins at basal conditions and prevents oxidative stress-induced up-regulation of bcl-2-like protein 11 (BIM). Accordingly, miR-221 protects differentiated DJ-1 knock-down ReNcell VM human dopaminergic neuronal cells from MPP^+-induced neurite retraction and cell death. DJ-1 is a known activator of the mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) pathway and may modulate miR-221 levels in part through this pathway. We found that inhibiting ERK1/2 decreases miR-221 levels, whereas over-expressing ERK1 in DJ-1 knock-down cells increases miR-221 levels. These findings point to a new cytoprotective mechanism by which DJ-1 may increase miR-221 expression through the MAPK/ERK pathway, subsequently leading to repression of apoptotic molecules. The inability of a pathogenic DJ-1 mutant to modulate miR-221 further supports the relevance of this mechanism in neuronal health and its failure in DJ-1-linked PD.

1. Introduction

Loss-of-function mutations in *DJ-1* (*PARK7*) are linked to autosomal recessively inherited Parkinson's disease (PD), and this gene is implicated in sporadic PD as well [1–3]. DJ-1 is a highly conserved homodimeric protein that is directly cytoprotective in neurons [4] and astroglia [5] under oxidative stress conditions [6,7]. Additionally, DJ-1 influences the transcriptome and proteome of a cell by regulating transcription factors and post-transcriptional processes, often interfering with pro-apoptotic processes [8,9] or upregulating antioxidant proteins [10,11].

microRNAs (miRNA) are short non-coding RNAs of about 22 nucleotides long that negatively regulate the post-transcriptional network by recognizing target mRNAs through base-pairing, and catalyzing transcript degradation or inhibiting mRNA translation [12]. Perturbations in miRNA expression have been implicated in a number of nervous system disorders including PD, suggesting that miRNA dysregulation is involved in the pathogenesis of these diseases [13].

DJ-1 can act as a direct transcriptional co-activator [14] and can modulate signal transduction to affect the transcriptome [15,16]. It may also have a role in direct RNA binding and post-transcriptional regulation [11]. In order to investigate miRNAs regulated by DJ-1, as well as their impact on the transcriptome and downstream processes involved in PD pathogenesis, we profiled miRNA expression in a human neuroblastoma cell model and found miR-221 to be down-regulated in DJ-1 knock down (KD) cells compared with controls. miR-221, one of

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the most abundant miRNA species in the human brain [17], has a critical role in cell survival [18], apoptosis [19–22], and neuritogenesis [23–25]. Interestingly, two separate miRNA profiling studies in PD patients have shown that decreased serum miR-221 may serve as a biomarker for diagnosis and disease stage evaluation [26,27]. In *in vitro* PD models, miR-221 was shown to regulate Transferrin receptor type 2 in SH-SY5Y cells challenged with the dopaminergic toxin 1-methyl-4-phenylpyridinium (MPP^+)[28], and to be protective by regulating PTEN in PC12 cells challenged with 6-hydroxydopamine [29]. Additionally, miR-221 is reported to be differentially expressed in the cingulate gyrus of PD patients and correlated with the downregulation of the expression of *SNCA, PARK2,* and *LRRK2*, genes linked to both autosomal recessive and dominant forms of PD [30,31].

Here, we report for the first time a mechanistic link between DJ-1 and mir-221. We found that knocking-down DJ-1 in a cellular model and knocking it out in the mouse brain result in down-regulation of miR-221. While re-introducing wild-type DJ-1 restores miR-221 expression, its PD-linked pathogenic M26I mutant fails to do the same. miR-221 mediates the cytoprotective activity of DJ-1 by repressing the expression of several pro-apoptotic proteins and protects differentiated ReNcell VM human dopaminergic neuronal cells from cell death and neurite retraction induced by MPP^+. DJ-1 may modulate miR-221 levels, in part, through the mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) pathway. These findings suggest that the modulation of miR-221 by DJ-1 is protective in the context of pathogenic mechanisms of PD including cell death [32] and neurite degeneration [33].

2. Results

2.1. miR-221 expression is modulated by wild-type DJ-1 but not its pathogenic mutant

To identify novel RNA transcripts regulated by DJ-1, we performed an Affymetrix Genechip® Human Gene 2.0 ST RNA expression microarray, which covered 40,716 RefSeq transcripts, to identify RNA species that are differentially expressed in a DJ-1 knock-down (KD) model of human dopaminergic neuroblastoma SH-SY5Y cells. A pool of four different small interfering RNAs (siRNAs) directed against DJ-1 was used to achieve potent and specific knock-down (Fig. 1**A**). Among the down-regulated transcripts, microRNA-221 (miR-221) exhibited the greatest fold reduction, with an expression level of 45% in DJ-1 KD cells compared to that in control cells.

This result from microarray analysis was confirmed by real time quantitative PCR (RT-qPCR) in both DJ-1 KD SH-SY5Y cells (Fig. 1**B**) and in the cerebral cortex of DJ-1 knock-out mice (Fig. 1**D–E**). In addition to down-regulation of miR-221 expression, DJ-1 KD in SH-SY5Y cells also decreased the precursor form of miR-221 (pre-miR-221), indicating that DJ-1 may regulate miR-221 prior to its processing to the mature form (Fig. 1**C**).

To examine the pathogenic consequence of DJ-1 in modulating miR-221 expression, the effect of wild-type and a PD-linked mutant DJ-1 were tested next. Over-expression of FLAG-tagged wild-type DJ-1 but not the empty control vector could re-constitute both mature miR-221 and pre-miR-221 levels in DJ-1 KD cells. However, FLAG-tagged pathogenic M26I mutant DJ-1 [34] could not rescue the down-regulation of miR-221 or pre-miR-221 caused by DJ-1 knock-down (Fig. 1**F–H**). This provides further evidence that DJ-1 may regulate miR-221 expression whereas PD-causing loss-of-function mutations in DJ-1 nullify its ability to regulate miR-221. This finding raises the possibility that miR-221 could be involved in a pathway that mediates a neuroprotective function of DJ-1.

2.2. miR-221 promotes cell survival under MPP^+ stress

DJ-1 is known to confer protection and increase cell survival against the dopaminergic neurotoxin MPP^+ [4,6,35,36]. To investigate whether miR-221 mirrors the cytoprotective effect of DJ-1, cells were made to over-express miR-221 by transfection with the precursor form of miR-221, pre-miR-221. They were then challenged with MPP^+ for 24 h, and survival was determined by MTS assay. Exogenous over-expression of miR-221 significantly increased cell survival under MPP^+ stress compared to cells transfected with scrambled pre-miR control (Fig. 2**A**). RT-qPCR confirmed increased levels of mature miR-221 in transfected cells (Fig. 2**B**). The functionality of miR-221 over-expression in these cells was verified by down-regulation of a known miR-221 target, fragile X mental retardation 1 (FMR1) mRNA [37] (Fig. 2**C**).

Conversely, when endogenous miR-221 was inhibited by anti-miR-221 (a single-stranded oligonucleotide that inhibits mature miR-221 by complementary binding) [22], cell survival decreased significantly in response to MPP^+(Fig. 2**D**). The effects of anti-miR-221 in reducing miR-221 levels (Fig. 2**E**) and up-regulating FMR1 mRNA levels (Fig. 2**F**) were confirmed. These findings show that miR-221 indeed mirrors the cytoprotective effect of DJ-1 and that a decrease of endogenous miR-221 renders cells more sensitive to toxic stress. That is, miR-221 at even endogenous levels is sufficient to be cytoprotective, suggesting that the modulation of miR-221 by DJ-1 may have physiological significance.

2.3. miR-221 targets transcripts of apoptotic proteins and rescues DJ-1 knock-down cells from cell death

One way by which DJ-1 confers cytoprotection is through interfering with apoptotic cascades and cell death processes [36,38–40]. To study whether miR-221 may be acting similarly, we used miR target prediction and database tools, TargetScanHuman [12,41], miRBase [42], and miRTarBase [43], to identify pro-apoptotic proteins that are predicted to be regulated by miR-221. Among targets with conserved sites predicted to pair with miR-221 seed sequence are apoptotic activators from the BCL2 family that have only the BH3 domain [44]: bcl-2-like protein 11 (BIM), bcl2 modifying factor (BMF), and bcl2 interacting protein 3-like (BNIP3L). Also predicted is forkhead box O3 (FOXO3a), a transcription factor implicated in the expression of genes necessary for cell death. Over-expression of miR-221 in SH-SY5Y cells significantly down-regulated the mRNA levels of all four targets implicated in promoting apoptosis, demonstrating that miR-221 may be acting at multiple levels to inhibit cell death (Fig. 3**A**). These apoptotic transcripts have been shown to be down-regulated in multiple cell types as well, such as BIM in PC12 cells [25], BMF in hepatocellular carcinoma cells [20], BNIP3L in 293T cells [45], and FOXO3a in human breast cancer cell lines [46].

Notably, miR-221 over-expression represses the protein expression of BIM, a pro-apoptotic protein shown to be crucial in neuronal apoptosis, particularly in the death of dopaminergic neurons in an *N*-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) model of PD [47,48]. miR-221 decreases the levels of all three pro-apoptotic BIM protein isoforms, BIM-short (BIM_S), BIM-long (BIM_L), and BIM-extra long (BIM_{EL}) (Fig. 3**B**), while miR-221 inhibition increases BIM protein levels (Fig. 3**C**). Remarkably, accumulation of BIM transcript following hydrogen peroxide (H_2O_2) treatment is completely prevented by miR-221 transfection (Fig. 3**D**).

As DJ-1 KD cells exhibit increased susceptibility to MPP^+ neurotoxicity compared to control cells [36], whether miR-221 could rescue this susceptibility was investigated next. First, cells were made to stably express non-targeting control (Ctrl) short hairpin RNA (shRNA) or pooled DJ-1 shRNA. Cells were then transduced with either lentiviral control miR (Ctrl miR) or miR-221 for 24 h. Stable DJ-1 KD led to decreased levels of mature miR-221, while transduction with lentiviral miR-221 robustly increased its levels (Fig. 3**E**). Next, cells were challenged with MPP^+ for 24 h, and cell death was assessed using lactate dehydrogenase (LDH) release. Stable DJ-1 KD cells, which exhibit a marked decrease in miR-221 levels, showed increased cell death in response to MPP^+ treatment. However, transduction with lenti-miR-

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